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MICRORNA-MEDIATED PLANT DEVELOPMENT AND RESPONSE TO ENVIRONMENTAL STRESS IN PERENNIAL GRASSES

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ABSTRACT

World population will pass nine billion by 2050, while the agricultural land area will not increase dramatically in the coming decades. To meet the ever-increasing food demand, genetically engineered crops have been rapidly adopted for crop productivity. MicroRNAs have become increasingly attractive as targets in crop genetic modification due to their regulatory role in fine-tuning many essential biological processes. My research explores the potentials of microRNA528 (miR528) and miR396 for use in genetic modifications of the important agronomic traits of plant development, abiotic stress response, and/or flowering time control in an economically and environmentally important perennial monocot species, creeping bentgrass (*Agrostis stolonifera*).

MiR528 is a conserved monocot-specific small RNA which is involved in multiple stress responses, however, experimental functional studies are lacking. In this study, we generated transgenic creeping bentgrass overexpressing a rice miRNA, *Osa-miR528*. Transgenic plants show altered plant development and enhanced salt and nitrogen (N) deficiency tolerance. The developmental changes include shortened internodes, increased tillers, and relatively upright growth. Enhanced salt stress tolerance is associated with improved water, chlorophyll, and potassium retention, cell membrane integrity, increased proline content, CATALASE activity, and reduced ASCORBIC ACID OXIDASE (AAO) activity; while improved N deficiency is associated with increased biomass, total N accumulation, chlorophyll synthesis, nitrite reductase activity, and reduced AAO activity. Molecular analysis identified AAO and COPPER ION BINDING1 as putative

targets of miR528 in creeping bentgrass. Both putative targets respond to salt stress and N deficiency.

The conserved miR396 is involved in plant vegetative and reproductive development and various environmental stress responses through targeting *Growth Regulating Factor* (*GRF*) transcription factor genes. In this study, we generated transgenic creeping bentgrass overexpressing a rice miRNA, Osa-miR396c. The transgenic plants exhibit altered development in both vegetative and reproductive growth, elimination of vernalization requirement, and enhanced salt stress tolerance. The altered plant development includes less shoot and root biomass, shorter internodes, smaller leaf area, fewer leaf veins and epidermal cells, and defects in filament elongation, anther dehiscence, and pollen viability. The enhanced salt tolerance is associated with improved water and chlorophyll retention, cell membrane integrity, and sodium ion exclusion during salt stress. The elimination of vernalization requirement is associated with the regulation of key genes *VRN1*, *VRN2*, and *VRN3* in the vernalization pathway. Four putative targets *AsGRF3-6* were identified in creeping bentgrass. These targets respond to high salinity, long-day photoperiod, and prolonged cold exposure. RNA-seq analysis shows that differentially expressed genes are involved in cell division, floral organ development, vegetative to reproductive transition, histone modification, oxidation reduction, environmental stress response, etc.

The study established the molecular pathways of miR528- and miR396-mediated plant salt stress and/or N deficiency tolerance in creeping bentgrass. It provides insight

into miRNA-mediated regulatory network in plant vegetative and reproductive development, abiotic stress response and flowering time control.

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CHAPTER I - LITERATURE REVIEW OF NEGATIVE REGULATORS OF MESSENGER RNA AND THE ROLE OF MICRORNA FOR PLANT GENETIC ENGINEERING

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1.1 Introduction

The world population is projected to surpass nine billion by 2050, which leads to rising demand for crop production due to the increasing consumption of crops, meat, diary, and biofuel. To meet the ever-increasing crop demands, global agriculture production may need to be increased by 100%-110% (Tilman, et al., 2011), whereas the agricultural land area is not expected to expand dramatically in the coming decades. Scientists have suggested that improving crop productivity, rather than expanding land area, is the most sustainable path to solve the issue (Foley, et al., 2011; Godfray, et al., 2010; Phalan, et al., 2011; Tscharntke, et al., 2012). Since genetically engineered crops were first commercialized 20 years ago, they have been rapidly adopted to improve crop yield. Globally, the land area for genetically engineered crops, mostly insect resistance or herbicide tolerance, has increased significantly from 1.7 million to over 175 million

hectares within 17 years (Clive, 2013; Privalle, et al., 2012). Currently, researchers and technology developers focus on a variety of crop traits, including enhanced tolerance to biotic and abiotic stresses, increased crop production, and improved nutritional composition (Wang, et al., 2003; Knauf, 1987; Lucca, et al., 2006). Genetically engineered crops with combined agronomic traits mentioned above are desirable, due to multiple stressors in the field where plants grow. Recently, negative regulators of messenger RNA (mRNA), especially microRNA (miRNA)-based genetic modifications, have emerged as an attractive technology for developing new crop cultivars with increased yields because miRNAs are involved in many key biological processes including response to environmental stimuli, defense against invading pathogens and plant development (Sun, 2012; Zhou and Luo, 2014; Pattanaik, et al., 2014; Seo, et al., 2013). In this chapter, I review the biogenesis and mechanisms of negative regulators of mRNA, which are two different classes of non-coding RNAs, encompassing miRNAs and small interfering RNAs (siRNAs). In addition, I highlight the most recent progress on miRNAs and miRNA-based gene silencing strategies in plant genetic modification.

1.2 Biogenesis and mechanisms of negative regulators of mRNA

In the early 1990s, the phenomenon of RNA silencing, which was known as co-suppression or posttranscriptional gene silencing, was discovered in plants (Napoli, et al., 1990; van der Krol, et al., 1990). By the end of the decade, more and more discoveries from diverse eukaryotic models linked small RNAs to gene silencing and collectively pointed that RNA silencing is an evolutionarily conserved mechanism (Fire, et al., 1998;

Hamilton and Baulcombe, 1999; Zamore, et al., 2000; Cogoni and Macino, 2000). A decade after the discovery of RNA silencing, distinct classes of endogenous small RNAs have been identified based on their different origins, which mainly include miRNAs and siRNAs (Chapman and Carrington, 2007; Llave, et al., 2002). Since then, a small RNA world has been gradually explored through understanding the depth and complexity of the regulatory role of these small RNAs. In this section, we will introduce the biogenesis and mode of actions of miRNAs and siRNAs in plants (Figure 1.1).

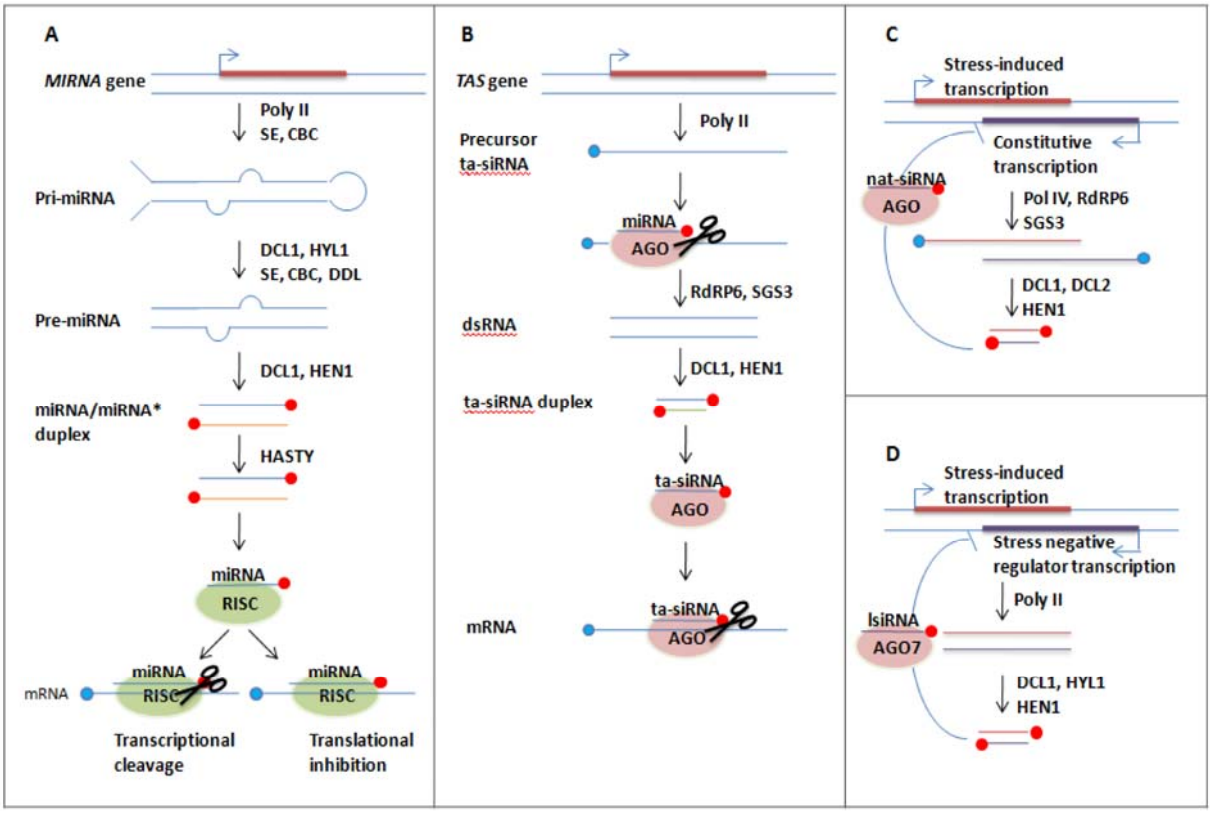

Figure 1.1 Biogenesis and mechanisms of negative regulators of mRNA in plants. A. MiRNA biogenesis and miRNA-mediated transcriptional cleavage and translational inhibition. B. Ta-siRNA biogenesis and ta-siRNA-mediated transcriptional cleavage. C. Nat-siRNA biogenesis and nat-siRNA-mediated cleavage of constitutively expressed transcript. D. A pathway of lsiRNA biogenesis and lsiRNA-directed mRNA degradation of stress negative regulator.

1.2.1 Biogenesis and mechanisms of miRNA

MiRNAs are regulatory small RNAs with the length of predominately 21 nt. They are transcribed from endogenous *MIRNA* loci to generate primary miRNAs (pri-miRNAs) by the help of Polymerase II (Kurihara and Watanabe, 2004; Aukerman and Sakai, 2003; Xie, et al., 2005). Then pri-miRNAs fold back to form hairpin-like structures and are processed into precursor miRNAs (pre-miRNAs). This process requires the nuclear cap-binding complex (CBC) and a C2H2-type zinc finger domain-containing protein SE to facilitate pri-miRNA biogenesis (Gregory, et al., 2008; Laubinger, et al., 2008; Grigg, et al., 2005; Lobbes, et al., 2006; Yang, et al., 2006); a forkhead-associated domain-containing protein DDL and a double-strand RNA-binding protein HYL1 to stabilize the foldback structure (Morris, et al., 2006; Vazquez, et al., 2004; Han, et al., 2004); and ultimately, another double-strand RNA-binding protein DCL1 to catalyze the excision of pri-miRNA to pre-miRNA (Kurihara, et al., 2006). Further, DCL1 processes pre-miRNA into approximately 21 nt miRNA/miRNA* double-strand duplex, to which is then added a methyl group at 3' terminal ribose by a small RNA methyltransferase HEN1 to protect miRNA from alternative end modification (Park, et al., 2002; Yang, et al., 2006). Next, the double-strand duplex is exported to the cytoplasm via the plant ortholog of exportin 5, HASTY (Bollman, et al., 2003). Finally, one functional miRNA strand of the duplex (either miRNA, miRNA*, or both) will be incorporated into the RNA induced silencing complex (RISC) to exert its function of post-transcriptional cleavage and/or translational inhibition (Brodersen, et al., 2008; Li, et al., 2013).

1.2.2 Biogenesis and mechanisms of siRNA

In contrast to miRNAs, which are derived from single transcripts with hairpin-shaped precursors, siRNAs are produced from the double-stranded RNA (dsRNA) duplexes (Voinnet, 2009). In addition, miRNAs regulate gene expression at post-transcriptional levels, whereas siRNAs act transcriptionally or post-transcriptionally through histone modification, DNA methylation, or mRNA degradation (Sunkar, et al., 2006; Sunkar and Zhu, 2007; Vaucheret, 2006; Baulcombe, 2004; Almeida and Allshire, 2005; Allshire, 2002). Since this chapter focuses on a negative regulator of mRNAs, we will discuss the classes of siRNAs which result in post-transcriptional gene silencing, including *trans*-acting siRNA (ta-siRNA), natural anti-sense siRNA (nat-siRNA), and long siRNA (lsiRNA).

The ta-siRNA precursor is transcribed from endogenous *TAS* loci and processed through ARGONAUTE (AGO) directed miRNA-guided cleavage. The single-stranded ta-siRNA precursor transcript is then processed in a dsRNA by RNA Dependent RNA Polymerase 6 (RdRP 6) and Suppressor of Gene Silencing 3 (SGS3), followed by further processing to generate phased 21-nt ta-siRNA duplexes by a member from the DCL family. Then one strand of the ta-siRNA duplex combines with AGO to trigger cleavage of the target RNAs due to the perfect sequence complementarity (Allen, et al., 2005; Gasciolli, et al., 2005).

Plant nat-siRNA is derived from a pair of convergently transcribed RNAs, which contain a constitutively expressed RNA and an environmental stress-induced RNA (Katiyar-Agarwal, et al., 2006; Borsani, et al., 2005). The overlapping region between the

sense transcript and the natural antisense transcript generate 21- to 24-nt nat-siRNA by the processing of DCL2 and/or DCL1, RdRP 6, SGS3 and Pol IV (Katiyar-Agarwal, et al., 2007; Katiyar-Agarwal, et al., 2006; Borsani, et al., 2005; Zhang and Trudeau, 2008). Then nat-siRNA triggers the cleavage of constitutively expressed sense transcript during environmental stresses.

In addition to nat-siRNA, another class of endogenous siRNA is lsiRNA, which is also derived from a convergently transcribed RNA pair and is stress-induced. During pathogen infection, lsiRNAs are produced as a defense mechanism to protect plant against the infection. Different from other siRNAs, lsiRNAs are 30 to 40-nt long (Katiyar-Agarwal, et al., 2007). Their biogenesis requires the involvement of DCL1, HYL1, HEN1 and AGO7, RDRP6, and Pol IV (Katiyar-Agarwal, et al., 2007). It has been proposed that lsiRNA destabilizes its targets through decapping and mRNA degradation (Katiyar-Agarwal, et al., 2007).

1.3 Roles of miRNA in plant genetic modification

The first miRNA lin-4, which controls the early developmental change through negatively regulation of the protein LIN-14, was discovered in *Caenorhabditis elegans* in 1993 (Lee, et al., 1993). Eight years later, many miRNAs were identified in both invertebrate and vertebrate species besides *Caenorhabditis elegans* (Lagos-Quintana, et al., 2001; Lau, et al., 2001; Lee and Ambros, 2001). Since then, miRNA-mediated post-transcriptional regulation has been recognized as a general mechanism. The discoveries prompted the identification of more miRNAs in various species. In the following year,

miRNAs were identified in distinct plant species, which includes rice and Arabidopsis (Llave, et al., 2002; Mallory, et al., 2002; Marker, et al., 2002; Reinhart, et al., 2002). Their important roles in plant morphology and development have also been unraveled (Reinhart, et al., 2002). Over the last decade, miRNAs have been attracting increased attention. MiRNA characterization indicates that it is an important regulatory element in various biological processes of plants, including plant growth, development, phase change, environmental stress response, and defense (Jones-Rhoades, et al., 2006; Chuck, et al., 2009; Liu and Chen, 2009; Chen, et al., 2004; Sunkar, et al., 2006). These regulatory miRNAs stimulate the idea of applying miRNA-based genetic modification technology for crop improvement. In this section, we summarize the most recent progress of plant trait modification with miRNA-mediated genetic engineering approaches (Table 1.1).

1.3.1 MiRNA-mediated plant organ development for plant genetic engineering

Increasing evidences shows that miRNAs regulate leaf and shoot development, root architecture, flower development, and vegetative to reproductive transition. All of these plant traits are critical for improving plant growth, and thereby contribute to increased plant biomass and yield. Here, we discuss each specific target trait in plant genetic modification (Figure 1.2).

1.3.1.1 Leaf development

Plant leaves convert light energy into chemical energy through photosynthesis, and can later provide the basis for plants growth and form the plant biomass. To improve crop yields and protect plant from environmental stressors which impair the photosynthetic

efficiency, it is important to understand the regulatory network during leaf growth and development, due to a complex interplay of multiple pathways (Kalve, et al., 2014). In Arabidopsis, overexpressing miR396 or reducing transcript levels of its targets, *GROWTH-REGULATING FACTORS* (*GRFs*), results in reduced leaf size (Kim and Kende, 2004; Kim, et al., 2003a; Liu, et al., 2009b). Conversely, overexpression of *GRFs* generates bigger leaves (Rodriguez, et al., 2010; Gonzalez, et al., 2010; Liang, et al., 2014). Recent findings suggest that GRF proteins work coordinately with GRF-interacting factors and chromatin-remodeling complexes to regulate leaf size (Debernardi, et al., 2014). High levels of miR319 leads to crinkled leaves in Arabidopsis (Efroni, et al., 2008; Koyama, et al., 2007; Palatnik, et al., 2003), while wider and thicker leaves in creeping bentgrass (Zhou, et al., 2013b). MiR319 controls leaf development through modulating its target *TCP4*, which is a negative regulator of cell proliferation in G2-M phase of the cell cycle (Schommer, et al., 2014). Additional analysis shows that *TCP4* regulates cell proliferation through activating different pathways, including a direct regulation on miR396b and the interaction between *TCP4* and *ICK1*/*KRP1*, a gene in the progression of cell cycle (Schommer, et al., 2014). Therefore, controlling miRNA expression in leaf development will generate plants with enlarged leaf area, and positively contribute to the increased plant biomass, especially when only harvesting the vegetative parts of plants for vegetables, forage crops, and biofuel crops.

Table 1.1 MiRNA/target module modification for improved agronomic traits.

MiRNA	Target(s)	Plant species	Agronomic trait	Modification strategy	Reference(s)
MiR396	GRF5	Arabidopsis	Biomass	Overexpression of *GRF5*	Gonzalez et al., 2010
MiR156	SPLs	Arabidopsis Rice Maize Switchgrass	Biomass	Overexpression of miR156	Schwab et al., 2005; Chuck et al., 2007; Fu et al., 2012; Xie et al., 2012
MiR156	SPLs	Arabidopsis	Trichome development	Overexpression of miR156	Yu et al., 2010
MiR171	LOMs	Arabidopsis	Trichome development	Overexpression of miR171-resistant *LOM*	Xue et al., 2014
MiR156	SPL14 SPL16	Rice	Grain yield	Overexpression of *SPL14* or *SPL16*	Miura et al., 2010 Wang eta l., 2012a
MiR397	LAC	Rice	Grain yield	Overexpression of miR397	Zhang et al. 2013b
MiR408	plantacyanin	Chickpea	Drought tolerance	Overexpression of miR408	Hajyzadeh et al., 2015
MiR394	LCR	Arabidopsis	Drought tolerance	Overexpression of miR394 or knockout *LCR*	Palatnik et al., 2003
MiR394	LCR	Arabidopsis	Salt tolerance	Overexpression of *LCR*	Palatnik et al., 2003
MiR319	TCPs	Rice	Cold tolerance	Overexpression of miR319 or knockdown *TCPs*	Yang et al., 2013b Wang et al,. 2014b
MiR398	CSD1 CSD2 CCS	Arabidopsis	Heat tolerance	Knockdown *CSD1*, *CSD2*, or *CCS*	Guan et al., 2013
MiR395	SULTR2;1 APS1 APS3 APS4	Rapeseed	Cadmium tolerance	Overexpression of miR395	Zhang et al., 2013a
MiR472	RPS5	Arabidopsis	Resistant to P. *syringae pv* tomato DC3000	miR472 loss of function mutant	Boccara et al., 2014
MiRNA PN-2013	MDHAR	Wheat	Resistant to wheat stripe rust	Knockdown *TaMDHAR*	Feng et al., 2014

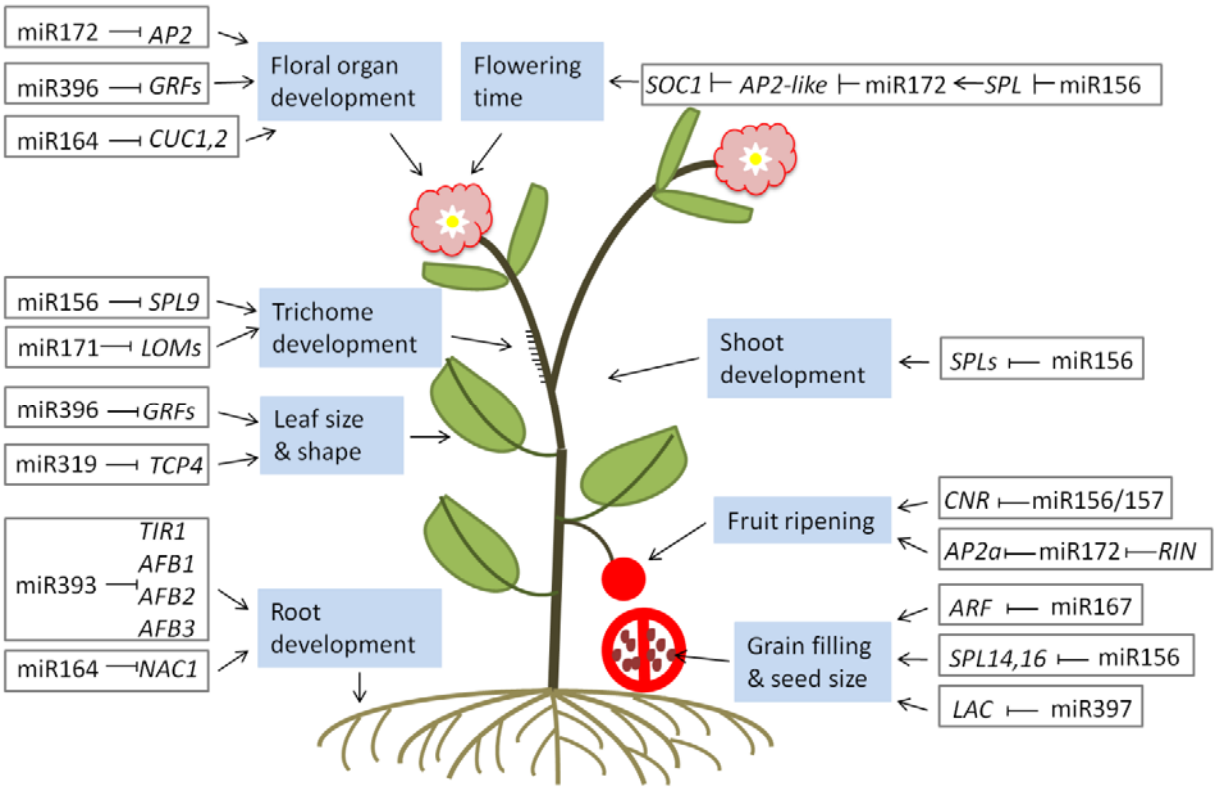

Figure 1.2 Summary of miRNA-target pairs-mediated plant organ development including both vegetative and reproductive stages.

1.3.1.2 Shoot development

Besides increased leaf size, another approach for high crop yield is the regulation of apical dominance to generate more shoots or leaves. MiR156 is involved in various aspects of plant development through regulating its targets *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) genes. SPLs are transcription factors, which control various developmental processes, including leaf and shoot development, phase change, and flowering time. Overexpression of miR156 reduces apical dominance and increases leaf amount and biomass in Arabidopsis (Schwab, et al., 2005). Similar results are also observed in transgenic rice, maize and switchgrass (Xie, et al., 2012; Fu, et al., 2012;

Chuck, et al., 2007). Transgenic switchgrass overexpressing pre-miR156 results in diverse phenotypes depending on the abundance of mature miR156. The plants with relatively low miR156 levels show moderately increased tiller numbers and biomass accumulation. As the amount of miR156 increases, transgenic plants display significantly more tillers and biomass yield, but a reduction in plant height. With the highest levels of miR156, transgenic plants exhibit severe dwarfism, increased tiller number, but reduced biomass yield (Fu, et al., 2012). The study suggests that miR156 breaks apical dominance and regulates tiller number in a dose-dependent manner. It is promising to adopt miR156-based genetic engineering strategy for improving plant biomass yield.

1.3.1.3 Trichome development

Trichomes are epidermal protuberances located on aerial parts of plant, that protect plant from excess transpiration, high temperature, radiation, UV light, and herbivore attack (Wagner, et al., 2004). Previous studies demonstrate that transcription factors R2R3 MYB, WD40 repeat protein, basic helix-loop-helix protein, and C2H2 zinc finger protein, and some phytohormones regulate trichome development (Serna and Martin, 2006; Ishida, et al., 2008; Yang and Ye, 2013; Wang and Chen, 2014). Recently, miR156 has been suggested to control the trichome development on stem and floral organ by modulating the expression of its *SPL* targets (Yu, et al., 2010). SPL9, one member of SPLs, directly promotes the transcription of *TCL1* and *TRY,* which are negative regulators of trichome development, therefore leading to fewer trichomes (Yu, et al., 2010). Consistent with this, constitutive expression of miR156 results in ectopic trichomes on the stem and floral organs, while overexpressing miR156-resistant forms of *SPLs* leads to

a reduction in trichome density. Overexpressing miR171 leads to fewer trichomes on stems and floral organs and further investigation uncovers interplay between miR156's targets *SPLs* and miR171's targets *LOMs* (Xue, et al., 2014). These findings highlight the complexity of the regulatory network that controls trichome development.

1.3.1.4 Root development

Roots are an indispensable organ for plant life because they anchor the plant in soil, and absorb water and nutrition for plant growth under both favorable and stress conditions. To better adapt to water or nutrient availability, plant root systems have great architectural plasticity through adjusting the length and amount of the lateral roots (Malamy, 2005; van der Weele, et al., 2000; Xiong, et al., 2006; Gruber, et al., 2013). During this process, the phytohormone auxin is essential in facilitating the lateral root initiation and elongation (Casimiro, et al., 2003; Perez-Torres, et al., 2008). In contrast, abscisic acid (ABA) functions in the opposite way, inhibiting the generation of lateral roots (Xiong, et al., 2006; Deak and Malamy, 2005; De Smet, et al., 2003). Recent findings indicate that miRNAs play pivotal roles in root development modulation. MiR393 is a conserved miRNA in many plant species. In Arabidopsis, miR393 has four validated targets, *TRANSPORT INHIBITOR RESPONSE 1* (*TIR1*), *AUXIN SIGNALING F-BOX 1* (*AFB1*), *AFB2*, and *AFB3*, which are auxin receptors (Jones-Rhoades and Bartel, 2004; Navarro, et al., 2006; Parry, et al., 2009). Constitutive expression of miR393 or its resistant targets leads to altered auxin responses in plant developmental processes (Chen, et al., 2011; Xia, et al., 2012). Recently, miR393 has been shown to regulate root architecture during nitrate responses through regulating its targets (Vidal, et

al., 2010). Another study indicates that the miR393/target module is involved in response to osmotic stress through repressing lateral root growth (Chen, et al., 2012). It is also proposed that miR393-mediated auxin signaling regulates root adaptation to drought stress (Chen, et al., 2012). In rice, miR393 has two targets, *OsTIR1* and *OsAFB2*, which are two orthologs of Arabidopsis *TIR1*. Transgenic rice overexpressing miR393 display altered primary and crown root growth through modulating auxin signal transduction, suggesting the conserved mechanisms of miR393-mediated regulation of root development (Bian, et al., 2012). In addition to miR393, miR164 also controls the lateral root density via regulating auxin signal pathway in Arabidopsis and maize (Li, et al., 2012b; Xie, et al., 2000). *NAC1*, the target of miR164, is a transcription factor gene that modulates auxin signaling to control the lateral root growth (Xie, et al., 2000). The role of phytohormone auxin in root development has been well studied for a long time. Recently identified miRNAs regulate plant root development through auxin signaling pathway. In addition to the miRNAs mentioned above, miR160, miR167, and miR390 also mediate root development via regulating auxin signal and their auxin receptor targets (Meng, et al., 2010). These studies suggest that regulating the levels of miRNAs in root development will benefit water and nutrient uptake, enhance plant resistance under stress conditions, and further improve plant growth and biomass.

1.3.1.5 Flower time and development

Flowering is a crucial phase to determine the plant reproduction success. Optimal flowering time provides favorable environmental conditions for seed development. Decades of physiological studies reveal that plant vegetative to reproductive transition

requires both environmental and endogenous cues. Environmental cues include temperature and photoperiod, while endogenous cues encompass autonomous promotion (independent of photoperiod), gibberellins, age, and carbohydrate availability (Kim, et al., 2009a; Wang, 2014; Albani and Coupland, 2010; Wahl, et al., 2013). The multiple flowering pathways are integrated to activate floral integrator and meristem identity genes initiating the transition from vegetative to reproductive growth. In temperate regions, many plant species, including both dicots and monocots, require the environmental cue of prolonged low temperature, also known as vernalization, to stimulate flowering. In fact, the actual flowering response requires another environmental cue of long daylength, which acts following vernalization. Together, they ensure plants flower in spring. Vernalization pathways have been proposed in both Arabidopsis and some crop species. In Arabidopsis, *FLOWERING LOCUS C* (*FLC*) is a strong floral repressor, which is epigenetically silenced by vernalization to trigger flowering (Bastow, et al., 2004). It also has been shown that *DCL1* and *DCL3* function redundantly to promote flowering by repressing *FLC* in Arabidopsis (Schmitz, et al., 2007), which suggests that miRNAs might be involved in plant vernalization pathway since *DCL1* is responsible for miRNA biogenesis and subject to negative feedback regulation via miR162 (Kurihara and Watanabe, 2004; Xie, et al., 2003). Recently, a newly identified pathway integrating age and vernalization cues is mediated by the abundance of miR172 and miR156 (Zhou, et al., 2013a). Both miR172 and miR156 are temporally regulated. Levels of miR156 are high in seedlings and decrease significantly with time, while the levels of its target *SPLs* increase in adult phase, resulting in the activation of miR172, and

thereby inhibiting the expression of *AP2-like* targets to release the repression on floral integrator *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*). In the study by Zhou *et al*., the polycarpic perennial species *C. flexuosa* does not respond to prolonged cold treatment until the plant is five week old. Overexpression of miR156 causes a prolonged juvenile phase, and prevents flowering in response to cold treatment. Conversely, low abundance of miR156 or *AP2-like* gene accelerates the transition to flower in response to vernalization. At the same time, another independent study also indicates that miR156 and miR172 play important roles in the age-dependent vernalization pathway in *A. alpine* (Bergonzi, et al., 2013). The advantage of the recruitment of age signals in response to winter cold or other cues is to ensure the full development of vegetative shoots and sufficient biomass.

Flower development consists of three phases, which include vegetative to reproductive transition, floral formation, and floral organ development. In addition to the floral transition phase, miRNAs are also implicated in floral formation and floral organ development. Luo *et al.* reviewed conserved miRNAs and their targets in the regulation of gene expression during flower development (Luo, et al., 2013). In the phase of floral organ formation, miR172 controls the inner floral whorl patterning in Arabidopsis, while it regulates lodicules development in rice and barley by targeting *AP2* gene. MiR164 is involved in sepal and petal boundary development, and carpel fusion through restricting its targets, the *NO APICAL MERISTEM*-related genes. In the phase of floral organ development, regulatory roles of miR159, miR160, miR166/165, miR167, miR169, and miR319 are discussed in the review (Luo, et al., 2013). In addition, several recent studies

show that miR396 plays important role in floral organ formation and development. Transgenic tobacco overexpressing *Populus trichocarpa miR396c* results in altered specification of floral organ type. The third and fourth floral whorls develop into stigmatoid anthers and fasciated styles, respectively (Baucher, et al., 2013). In Arabidopsis, miR396 controls carpel number and pistil development via regulating its *GRF* targets, which are highly expressed in developing pistils (Liang, et al., 2014). In rice, miR396d and its *GRF* targets are reported to function in floral organogenesis through regulating *OsJMJ706* and *OsCR4*. Transgenic rice overexpressing miR396d leads to open husks and long lemmas, which largely impact grain yields (Liu, et al., 2014).

1.3.1.6 Fruit ripening

Fruit ripening softens fruit tissues to promote seed dispersal. Additionally, fruit traits including color, size, texture, flavor, and mature time impact the interest and use of fruits by humans. Ripening is a complex and highly coordinated process which requires the involvement of hundreds to thousands of genes. Fruit traits are frequent targets for modifications in physiological and molecular studies. Tomato, the important horticultural crop, is the most studied model for fruit development and ripening. Through identification and characterization of ripening-associated mutants in tomato, many transcription factor genes are found in the regulation of fruit ripening, such as *RIPENING INHIBITOR* (*RIN*), *COLOURLESS NONRIPENING* (*CNR*), *NONRIPENING* (*NOR*), *APETALA2a* (*AP2a*), and *FRUITFULL 1* (*FUL1*) (Vrebalov, et al., 2002; Tigchelaar, et

al., 1973; Manning, et al., 2006; Karlova, et al., 2011; Chung, et al., 2010; Bemer, et al., 2012).

Many genes encoding transcription factors are targets of miRNAs. MiRNA-target pairs in tomato fruit are identified through degradome sequencing (Karlova, et al., 2013). The result shows that 56 mRNAs encoding for transcription factors among the total of 119 miRNA-mRNA target pairs, such as miR156/157-*CNR* and miR172-*AP2a* pairs, indicating the importance of miRNAs in fruit development (Karlova, et al., 2013). Later, Chen *et al.* experimentally confirmed that miR156 and miR157 were involved in modulating tomato ripening and softening through targeting *CRN* (Chen, et al., 2015). High abundance of miR157 leads to delayed ripening. Instead of affecting the onset of ripening, miR156 controls fruit softening (Chen, et al., 2015).

As post-transcriptional regulators, miRNAs are also regulated by transcription factors at transcriptional level through regulating the genes that encode miRNAs (Baek, et al., 2013; Yant, et al., 2010). A recent study shows that RIN, a vital transcription factor, which directly targets ripening-related transcription factors, also regulates miR172a through directly binding of its promoter region (Gao, et al., 2014). MiR172 is involved in fruit ripening via repressing the important fruit ripening regulator *AP2a*. The identification of RIN-miR172 regulation adds another layer of regulation in miRNA-mediated tomato fruit ripening.

1.3.1.7 Seed development

Seed development is a major focus of crop breeders, since it is directly associated with grain yield. Emerging evidence shows that miRNAs act as important regulators of

seed development. Small RNA high-throughput sequencing analysis shows the increasing expression of the most known miRNAs as rice grain filling goes on (Peng, et al., 2013). Recent deep sequencing analysis compared the differential expression of miRNAs in rice superior and inferior spikelets (Peng, et al., 2014). In general, superior spikelets had faster grain-filling during seed development and produced high quality seeds compared to inferior spikelets. The result indicates that among differentially expressed miRNAs, most of them with higher expression levels are associated with the superior instead of the inferior spikelets during grain filling (Peng, et al., 2014).

Besides, studies of certain specific miRNAs also suggest a high correlation with seed development. Overexpression of miR167 leads to reduced tiller number, spikelet number, and grain filling rate through targeting *ARF* (Liu, et al., 2012). A proposed regulatory model of miR167 in rice grain filling shows that the grain filling rate is positively regulated by auxin content, which is positively correlated with *ARF* , the target of miR167 (Xue, et al., 2009). In addition, overexpression of miR393 or miR172 results in reduced seed weight (Zhu, et al., 2009; Bian, et al., 2012). *OsSPL14* and *OsSPL16*, the two targets of miR156, positively regulate grain yield in rice (Miura, et al., 2010; Wang, et al., 2012a). MiR397 is the first experimentally confirmed positive regulator of miRNA in seed size and grain yield (Zhang, et al., 2013b). OsmiR397, which is highly expressed in rice young panicles and grains, increases grain yield via repressing its target *OsLAC*, a gene encoding L-ascobate oxidases (Zhang, et al., 2013b). A similar role of miR397 is also observed in transgenic Arabidopsis overexpressing miR397, which includes increased inflorescence shoots, silique number and length, seed numbers, and seed size

(Wang, et al., 2014a). Due to the conserved regulatory mechanism of miR397 in controlling grain yield through targeting laccase genes in both monocot and dicot species, there is potential to facilitate genetic engineering of other crop species with greater grain yield.

1.3.2 MiRNA-mediated plant response to abiotic stress for plant genetic engineering

As sessile organisms, plants cannot escape from environmental stimuli, such as water stress, high soil salinity, extreme temperature, light stress, oxidative stress, nutrition limitation, and heavy metal stress. The on-going change in climate conditions and ever-increasing world population exaggerate water scarcity, temperature rise, as well as increase water, soil, and air pollution. To adapt, survive, and reproduce under these unfavorable environmental conditions, plants have evolved a multitude of abiotic stress responses, including physiological and molecular processes. Understanding the molecular mechanisms in plant response to abiotic stress is important to develop stress-tolerant crops with genetic engineering technologies. Currently, many downstream functional proteins and upstream regulatory proteins, which are involved in plant abiotic stress responses, have been characterized, but understanding the regulatory mechanisms in plant response to abiotic stress remains elusive. Additionally, increasing evidence suggests that miRNAs serve as important post-transcriptional regulators in plant response to various environmental stresses, which add a layer of complexity to the regulatory networks. In this section, we summarize the most recent progress of genetically engineered plants with enhanced stress tolerance via modification of miRNA-target nodes and promising transformative tools to adapt crops to abiotic stress (Figure 1.3).

1.3.2.1 Drought stress

MiR408 is an evolutionarily conserved miRNA regulated by dehydration and mechanical stresses (Kantar, et al., 2010; Lu, et al., 2005). A recent study in chickpea experimentally confirms its role in plant response to dehydration (Hajyzadeh, et al., 2015). Overexpression of miR408 results in enhanced drought stress tolerance via direct regulation of its target *plantacyanin*, a copper ion binding protein (Hajyzadeh, et al., 2015), which suggests an improved capability in maintaining copper homeostasis in response to drought stress. In addition, the expression levels of drought responsive genes *DREB1A*, *DREB2A*, *RD17*, *RD29A*, and *RD22* are induced in transgenic chickpea overexpressing miR408 in comparison with the vector control plants during drought stress conditions. The result indicates a crosstalk between miR408 and other drought responsive elements and they might act coordinately for enhanced drought stress tolerance. Besides enhanced drought tolerance, transgenic chickpea overexpressing miR408 also leads to a pleiotropic effect of a dwarf phenotype. The result indicates that there must be other targets of miR408, which acts as a growth rate regulator. In the process of crop genetic engineering, it is critical to avoid the pleiotropic effect of miRNAs by detecting and modulating the specific target genes with desirable agronomic traits.

The role of miR164 has been well studied in the regulation of plant growth and development through regulating five NAC transcription factor genes *CUC1*, *CUC2*, *NAC1*, *At5g07680*, and *At5g61430* in Arabidopsis (Rhoades, et al., 2002; Laufs, et al.,

2004; Mallory, et al., 2004; Guo, et al., 2005; Kim, et al., 2009b). Several studies show that miR164 is also involved in plant response to abiotic stress, but the underlying molecular mechanism is not well understood until the recent characterization of *Oryza miR164-targeted NAC* (*OMTN*) genes (Fang, et al., 2014). Transgenic rice overexpressing *OMTN2*, *OMTN3*, *OMTN4*, or *OMTN6* exhibit increased drought sensitivity in the reproductive stage, which is associated with the reduced relative spikelet fertility (Fang, et al., 2014). Further genomic expression profile analysis suggests that overexpression of miR164 targets *OMTNs* causes the reduction of many regulatory and functional genes in response to drought stress (Fang, et al., 2014). The study establishes the connection between miR164 and abiotic stress response. It also provides the possibility of manipulating *OMTNs* for enhanced drought tolerance in rice and other crop species.

1.3.2.2 Salt stress

MiR394 is involved in plant abiotic stress response via silencing its target gene *LEAF CURLING RESPONSIVENESS* (*LCR*). Transgenic Arabidopsis constitutively expressing miR394 and *LCR* loss of function mutant (*lcr*) plants are sensitive to salinity, whereas overexpression of *LCR* result in enhanced salt tolerance compared to wild type controls (Palatnik, et al., 2003). Interestingly, miR394 overexpression plants and *lcr* mutants are resistant to drought stress, whereas transgenic plants overexpressing *LCR* are supersensitive to drought stress (Palatnik, et al., 2003). In addition, miR394 positively regulates ABA- and stress- responsive genes *ABI3*, *ABI4*, *ABI5*, *ABF3*, *ABF4*, *RD29A*, *KIN1*, and *RD22*. It is well known that plants adopt the similar strategies of osmotic

adjustment in response to drought and salt stresses. The distinct mechanisms in response to drought and salt stress are proposed to be regulated by a functional balance of miR394-*LCR* pairs. It is also plausible that miR394 regulates other direct or indirect targets which are involved in the salinity-specific adjustments of salt compartmentalization and excretion against ionic disequilibrium. Further investigation of the miR394-mediated molecular mechanisms is necessary when applying the strategy of manipulating mi394 or its targets for specific trait.

1.3.2.3 Cold stress

MiR319 is a conserved miRNA, which targets *TEOSINTE BRANCHED*/*CYCLOIDEA*/*PCF* (*TCP*) transcription factor genes (Palatnik, et al., 2007; Koyama, et al., 2007; Ori, et al., 2007; Koyama, et al., 2010; Schommer, et al., 2008; Schwab, et al., 2005) Koyama et al., 2010). It has been shown to be involved in cold stress response in Arabidopsis, rice, and sugarcane (Liu, et al., 2008; Zhou, et al., 2010; Thiebaut, et al., 2012). Recent studies illustrate that overexpression of miR319b leads to enhanced cold stress tolerance in transgenic rice through directly repressing its targets *OsPCF5*, *OsPCF8*, *OsPCF6*, and *OsTCP21* (Yang, et al., 2013; Wang, et al., 2014b). In addition, RNAi transgenic plants with down-regulation of *OsPCF5*, *OsPCF8*, *OsPCF6*, or *OsTCP21* show enhanced cold tolerance; while overexpression of *OsPCF6* or *OsTCP21* significantly decreases plant cold resistance partially due to their negative regulation in ROS generation or scavenging (Wang, et al., 2014b; Yang, et al., 2013). Besides the direct targets of miR319, it also positively regulates the expression of cold-responsive genes, including *DREB1A/B/C*, *DREB2A*, and *TPP1/2*, which contribute to the

enhanced cold tolerance as well (Wang, et al., 2014b). It is likely that miR319-mediated cold tolerance requires coordinated regulation of multiple cold-responsive pathways.

1.3.2.4 Heat stress

MiR159 is a conserved plant miRNA that regulates the cleavage of *GAMYB* genes. In wheat, *TaGAMYB1* and *TaGAMYB2* are identified as targets of miR159 (Wang, et al., 2012b). Wheat TamiR159 has been reported to be down-regulated during heat stress (Wang, et al., 2012b). In addition, transgenic rice overexpressing *TamiR159* is more sensitive to heat (Wang, et al., 2012b). In Arabidopsis, its targets are the *GAMYB*-like genes *AtMYB33* and *AtMYB65*, which are functionally redundant. Arabidopsis *myb33mby65* double mutants show sensitivity to heat stress (Wang, et al., 2012b). The results indicate that the miR159/*GAMYB* module might participate in a heat-related pathway. Interestingly, overexpression of *TaGAMYB1* does not show improved heat stress tolerance (Wang, et al., 2012b). It is plausible that constitutive expression of *TaGAMYB1* induce other factors that override the effects of heat tolerance. In addition, identification and characterization of other targets of TamiR159 in response to heat stress would provide better understanding of miR159-mediated heat tolerance.

MiR398 is rapidly induced under heat stress, while its targets CSD1, CSD2, and CCS are reduced in response to heat in Arabidopsis (Guan, et al., 2013). Mutant plants *csd1*, *csd2*, and *ccs* exhibit enhanced heat tolerance compared to wild type controls (Guan, et al., 2013). In addition, constitutive expression of the miR398-resistant form *CSD2* results in enhanced sensitivity to heat compared to the plants overexpressing normal *CSD2*, which is associated with reduced levels of heat stress transcription factors and heat shock

proteins (Lu, et al., 2013). Considering that miR398 and its targets are highly conserved across various plant species, manipulating miR398-targeted *CSD2* might be a viable strategy for breeding heat-tolerant crops.

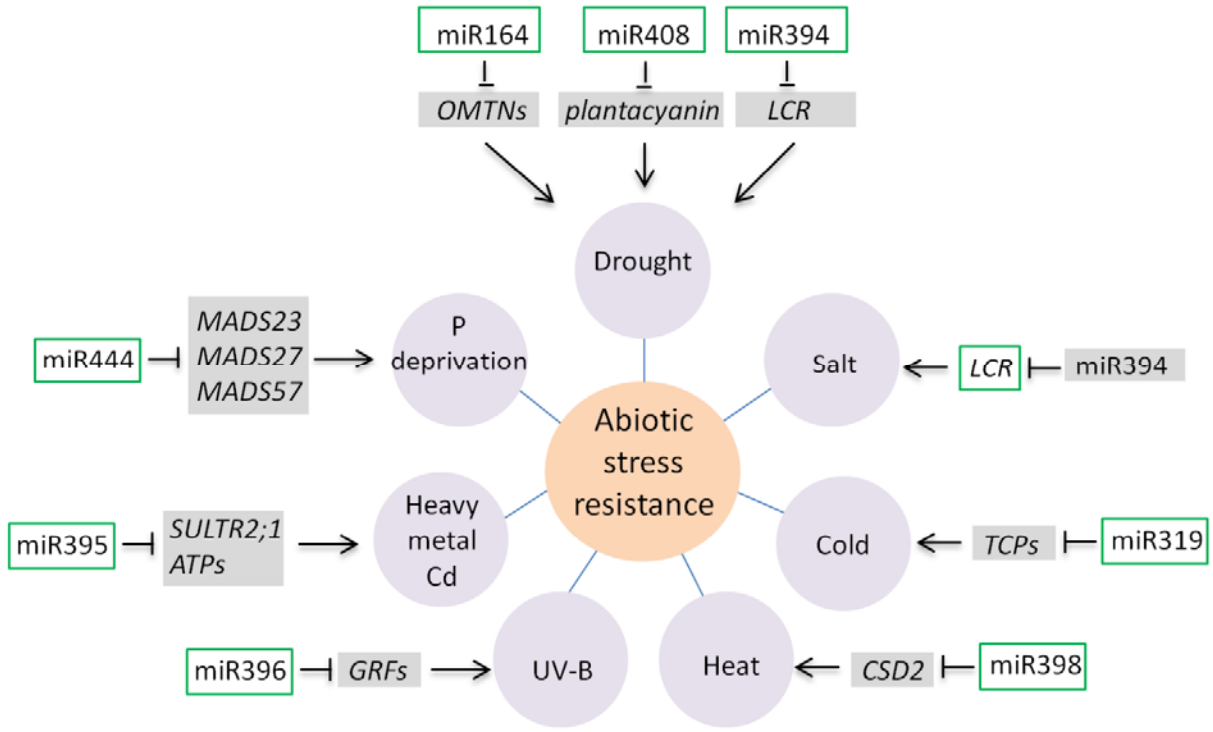

Figure 1.3 Summary of modifications of miRNA-target pairs with enhanced abiotic stress resistance. Negative regulators (miRNAs or target mRNAs) in response to abiotic stress are labeled with gray background; positive regulators in response to abiotic stress are bordered.

1.3.2.5 UV-B radiation

Plant exposure to UV-B radiation causes inhibition in cell proliferation or/and cell expansion (Kakani, et al., 2003; Hectors, et al., 2007; Wargent, et al., 2009; Robson and Aphalo, 2012). It is known that miR396 controls leaf cell proliferation and expansion via regulating *GRFs* in leaf primordia. A recent study demonstrated the role of

miR396/GRFs module in UV-B-mediated leaf growth inhibition in Arabidopsis (Casadevall, et al., 2013). The study illustrates that UV-B radiation induces the expression of miR396, while it reduces transcripts of GRF1, GRF2, and GRF3 in proliferating tissues (Casadevall, et al., 2013). Further analysis indicates that the induction of miR396 is mediated by mitogen-activated protein kinase 3, which is activated by UV-B radiation. Transgenic plants expressing reduced endogenous miR396 through artificial target or expressing miR396-resistant *GRFs* display reduced sensitivity to UV-B-mediated growth inhibition (Casadevall, et al., 2013). The results suggest that modulating the levels of miR396 or *GRFs* can be applied in crop species for enhanced UV-B radiation tolerance, contributing to improved crop yields.

1.3.2.6 Heavy metal stress

Plants require essential metals such as iron, copper, zinc, and manganese as nutrients, and components for various enzyme and protein reactions. However, overload of these essential components or uptake of non-essential toxic metals such as cadmium (Cd), lead, and mercury result in detrimental effects for plants. To minimize the heavy metal stress, the first step is to dissect the toxic metal-responsive genes and the regulation pathways. Increasing evidence shows that miRNAs and their targets are involved in heavy metal stress response. Yang *et al.* reviewed the recent advances in the identification of miRNAs and their targets and defined their potential roles in plant tolerance to heavy metals (Yang, 2013).

A recent study shows that genetically engineered rapeseed (*Brassica napus*) with modified miR395 displays enhanced Cd stress tolerance (Zhang, et al., 2013a). Cd

contamination becomes a severe environmental stress worldwide due to its significant release via anthropogenic activities. Accumulation of Cd in plants disrupts the enzyme activity, represses cell division and root growth, and consequently affects crop production and human health via the food chain (Chaney, et al., 1999; Clemens, 2006; Chen, et al., 2009). MiR395 is a conserved miRNA that regulates plant sulfate assimilation and distribution through regulating sulfate transporter gene *SULTR2;1* and ATP sulphurylases genes *APS1*, *APS3*, and *APS4* (Kawashima, et al., 2009; Liang, et al., 2010). In rapeseed, miR395 is induced under Cd stress (Zhang, et al., 2013a). Transgenic rapeseed overexpressing miR395 shows reduced oxidative stress damage, and increased chlorophyll and glutathione contents in comparison with wild type controls (Zhang, et al., 2013a). Under Cd stress, transgenics accumulate more sulfur, Cd and biomass than wild type controls, but exhibit repressed Cd translocation in plants. Further molecular analysis reveals that miR395 positively regulates the metal-tolerance genes *BnPCS1*, *BnHO1* and *Sultr1;1* (Zhang, et al., 2013a). A previous study proposed one mechanism for plant tolerance to Cd, which is metal-chelating through the interaction between Cd and sulfur-containing compounds (Khan, et al., 2008). In addition, some sulfate transporters are regulated by heavy metals (Xue Mei, et al., 2007; Huang, et al., 2010). Therefore, it is plausible that miR395-mediated sulfate assimilation is involved in the signal pathway of synthesis of sulfur-containing components, and consequently contributing to Cd detoxification in plants.

1.3.2.7 Nutrition deprivation

Nitrogen (N) and phosphorus (P) are two of the most essential macronutrients for plant growth, development, and reproduction. The monocot-specific small RNA miR444 is reported to be involved in nitrate accumulation and phosphate-starvation responses in rice (Yan, et al., 2014). Four targets of miR444 have been confirmed in rice, which are the MIKC-type MADS-box genes *OsMADS23*, *OsMADS27a*, *OsMADS27b*, and *OsMADS57* (Sunkar, et al., 2005; Lu, et al., 2008; Wu, et al., 2009; Li, et al., 2010). They are homologous to Arabidopsis *ANR1*, a key component in the nitrate signaling pathway, which regulates lateral root growth. The abundance of rice miR444 is positively regulated under N- and P-starvation conditions (Yan, et al., 2014). Overexpression of miR444 enhances nitrate accumulation in rice under normal growth conditions, but not under N-limiting conditions (Yan, et al., 2014). Gene expression analysis shows that the transcripts of four nitrate transporters are induced in miR444-overexpressing rice (Yan, et al., 2014). However, nitrate remobilization from old to young leaves under N-limiting conditions is disrupted in the transgenic rice, and thereby enhancing the sensitivity of miR444 transgenic plants to N-limiting conditions (Yan, et al., 2014). Interestingly, miR444 overexpression in rice causes root architectural changes under phosphate-starvation, increased phosphate accumulation under different concentrations of phosphate supply, and the induction of phosphate transporter genes (Yan, et al., 2014). The study indicates that miR444 plays multiple roles in nitrate signal pathway and phosphate-starvation responses in rice. In order to engineer the traits for enhanced N- and P-starvation tolerance, thorough dissection and manipulation of miR444 targets is a more practical approach.

In addition to miR444, other miRNAs also play important functions in response to nutrient deficiencies. For instance, miR395 regulates sulfur distribution and homeostasis (Kawashima, et al., 2011), miR399 is involved in phosphate-starvation signaling (Chiou, et al., 2006), miR393 and miR167 act in the nitrate-signaling pathway (Gifford, et al., 2008; Vidal, et al., 2010), miR169 is involved in N-starvation response (Zhao, et al., 2011).

1.3.3 MiRNA-mediated plant response to biotic stress for plant genetic engineering

When plants are subjected to bacterial, virus, fungi, insect, or nematode infections, they are able to respond at the molecular level through rapidly regulating expression levels of stress-responsive genes. The role of miRNAs in this complicated defense mechanism was not discovered until a decade ago (Llave, 2004). Currently, studies are mainly focusing on identification and characterization of biotic stress-responsive miRNAs and their targets. In this section, we discuss the responses of miRNAs to different types of biotic stresses (Figure 1.4).

1.3.3.1 Bacteria

Since the identification of miR393 which was implicated in plant response to bacterial infection by repressing auxin signaling, accumulating evidence suggests that miRNAs are involved in immune responses (Navarro, et al., 2006; Navarro, et al., 2008; Jagadeeswaran, et al., 2009). MiR472 targets the disease resistance genes, which belong to the coiled-coil nucleotide-binding leucine-rich-repeats family (CNL). A recent study demonstrates that Arabidopsis miR472 and a key RNA silencing factor RDRP6 act coordinately in the regulation of immune responses (Boccara, et al., 2014). In the study,

miR472 loss of function mutant plants exhibit enhanced resistant to *P. syringae pv* tomato DC3000, whereas miR472-overexpressing plants are more susceptible to the bacterial strain.

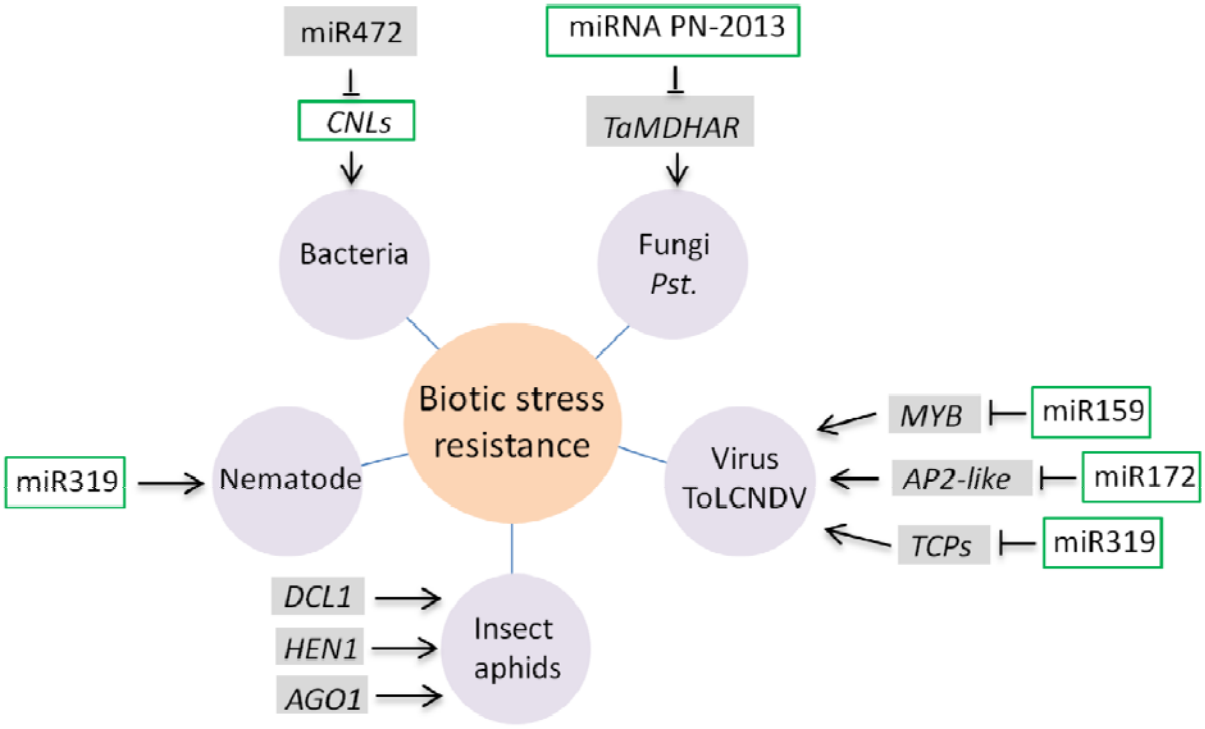

Figure 1.4 Regulatory roles of miRNAs, miRNA-target pairs, and key genes in miRNA biogenesis pathway in response to biotic stress resistance. Negative regulators in response to biotic stress are labeled with gray background; positive regulators in response to biotic stress are bordered.

1.3.3.2 Fungi

Wheat stripe rust is one of the most destructive fungal diseases caused by *Puccinia striiformis* f. sp. *tritici* (*Pst*). A recent study demonstrated that a novel wheat miRNA PN-2013-targeted *TaMDHAR* gene is involved in the response to *Pst* at wheat seedling stage (Feng, et al., 2014). *TaMDHAR* plays a vital role in the regulation of ROS metabolism through the ascorbate-gluthione cycle (Arrigoni, et al., 1981). Knockdown mutants of

TaMDHAR exhibit enhanced resistance to wheat stripe rust (Feng, et al., 2014). Gene expression analysis shows that pathogenesis-related genes are induced in *TaMDHAR* mutants, which may contribute to the improved resistance to *Pst*. Detailed dissection of the regulatory mechanism of PN-2013 in response to *Pst* would provide a better understanding of pathogen-host interactions, and therefore pave the way for engineering miRNA for enhanced pathogen resistance.

1.3.3.3 Virus

Tomato leaf curl New Delhi virus (ToLCNDV) infects tomato and other crops with the symptom of upward leaf curling and results in severe yield loss. The accumulation of miR159, miR319, and miR172 is observed as the ToLCNDV agroinfection progression in tomato cv Pusa Riby, tomato cv JK Asha, and chilli plants (Naqvi, et al., 2010). The study revealed that these miRNAs might be responsible for the leaf curl symptom and have the potential to serve as biomarkers for ToLCNDV infection. A recent study identified several novel miRNAs (Tom_4; Tom_14; Tom_17; Tom_21; Tom_29; Tom_43) and their targets in response to ToLCNDV infection (Pradhan, et al., 2015), which could allow better understanding for the miRNA-mediated ToLCNDV-tomato interaction.

Recently, studies are mainly focused on identification of miRNAs in response to viral infection (Pacheco, et al., 2012; Amin, et al., 2011), while the direct transgenic approaches using plant endogenous miRNAs for promoting antiviral resistance are lacking. However, the strategy of engineering artificial miRNAs, which target viral

genome for antiviral resistance, becomes an effective RNA silencing tool *in planta* (Ai, et al., 2011; KUNG, et al., 2012; Fahim, et al., 2012; Zhang, et al., 2011).

1.3.3.4 Insect

A recent report links miRNA-mediated secondary metabolic defense pathway to aphid pest resistance (Kettles, et al., 2013). In the study, deficient processing of miRNAs in *dcl1*, *hen1*, or *ago1* mutants significantly decreases the offspring of green peach aphids, partially due to the enhanced production of camalexin, which largely impacts reproductive ability of aphids (Kettles, et al., 2013). Detailed investigation demonstrates that *dcl1* mutants promote the production of camalexin through inducing the key genes in the camalexin biosynthetic pathway during aphid attack (Kettles, et al., 2013). In addition, the defense against attack from chewing herbivores has also been characterized in siRNAs and artificial small RNAs (Guo, et al., 2014; Pandey, et al., 2008; Pandey and Baldwin, 2007), which provides a variety of promising and preferable small RNA-mediated defense strategies for engineering plants resistant to plant-infesting insects.

1.3.3.5 Nematode

Deep sequencing analysis of miRNAs in soybean has been conducted to identify soybean cyst nematode (SCN)-responsive miRNAs (Li, et al., 2012). The result indicates that 101 miRNAs shows differential expression in response to the nematode infection, including conserved stress-responsive miRNAs, such as miR319, miR169, and miR390 (Li, et al., 2012). Currently, information on nematode virulence is still in its infancy. Further genome-wide sequencing analysis to identify nematode-responsive miRNAs in

diverse plants and characterization of SCN-responsive miRNA/target modules will boost our understanding of the molecular mechanism of nematode infection.

1.4 MiRNA-based gene silencing strategies in plant genetic modification

Since the discovery of homology-dependent gene silencing phenomena in plants, also termed as RNAi, it has become an efficient tool for the functional characterization of genes through knocking down the expression of an individual targeted gene, or multiple gene family members. One of the RNAi phenomena is co-suppression, which can be used to introduce the sense transgene into plants to reduce or delete the transcripts of both transgene and the endogenous homologous existing in the plant genome (de Carvalho Niebel, et al., 1995). Antisense suppression describes another RNAi phenomenon, in which antisense strand of targeted mRNA can be introduced into plants and forms an mRNA duplex to block translation. Later, a highly efficient post-transcriptional gene silencing construct, termed hairpin RNA interference (hpRNAi), was been designed. It encodes a self-complementary hairpin RNA containing sense and anti-sense arms, and an intron for enhancing the silencing efficiency (Wesley, et al., 2001).

In addition, given the importance and wide-range of miRNAs, many strategies have also been designed to repress the expression of these regulatory molecules for better understanding of specific miRNA-target pairs. Artificial miRNA (amiRNA) technology is used to directly repress the expression of a specific miRNA or a whole miRNA family. If the amiRNA targets a mature miRNA sequence, all miRNA family members will be silenced. If the amiRNA targets the stem-loop region of a pre-miRNA, only this individual miRNA can be silenced (Eamens, et al., 2011). Another approach to inhibit the

activity of miRNA is the target mimicry, in which the modified miRNA contains an uncleavable target site and thus, leads to the non-productive interactions (Franco-Zorrilla, et al., 2007). Alternatively, the artificial target approach, which introduces miRNA-resistant forms of the targets into the plant genome, results in the disturbed miRNA activity (Reyes and Chua, 2007).

1.5 Concluding remarks

A new layer of gene regulatory networks have been gradually unveiled since the discovery of a hidden miRNA world. In this chapter, we have summarized the involvement of miRNAs in plant development, abiotic, and biotic stress responses, the promising approaches for miRNA-mediated plant genetic engineering, as well as miRNA-based gene silencing strategies for characterization of gene function and plant genetic modification. The rapid development of high-throughput sequencing tools and the large amount of available genomic data will boost the progress for miRNA functional investigations. A thorough understanding of the action of miRNA-target pairs will lay the foundation for elucidating the complex regulatory networks implicated in all aspects of plant life processes, facilitating in developing novel strategies for improving a multitude of plant traits.

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References

Ai T, Zhang L, Gao Z, Zhu C, Guo X (2011) Highly efficient virus resistance mediated by artificial microRNAs that target the suppressor of PVX and PVY in plants. Plant Biology **13:** 304-316

Albani MC, Coupland G (2010) Chapter eleven-comparative analysis of flowering in annual and perennial plants. Curr Top Dev Biol **91:** 323-348

Allen E, Xie Z, Gustafson AM, Carrington JC (2005) microRNA-directed phasing during trans-acting siRNA biogenesis in plants. Cell **121:** 207-221

Allshire R (2002) Molecular biology. RNAi and heterochromatin--a hushed-up affair. Science **297:** 1818-1819

Almeida R, Allshire RC (2005) RNA silencing and genome regulation. Trends Cell Biol **15:** 251-258

Amin I, Patil BL, Briddon RW, Mansoor S, Fauquet CM (2011) A common set of developmental miRNAs are upregulated in *Nicotiana benthamiana* by diverse begomoviruses. Virol J **8:** 300

Arrigoni O, Dipierro S, Borraccino G (1981) Ascorbate free radical reductase, a key enzyme of the ascorbic acid system. FEBS Lett **125:** 242-244

Aukerman MJ, Sakai H (2003) Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes. Plant Cell **15:** 2730-2741

Baek D, Park HC, Kim MC, Yun D (2013) The role of Arabidopsis MYB2 in miR399f-mediated phosphate-starvation response. Plant Signal Behav **8:** 362-373

Bastow R, Mylne JS, Lister C, Lippman Z, Martienssen RA, Dean C (2004) Vernalization requires epigenetic silencing of FLC by histone methylation. Nature **427:** 164-167

Baucher M, Moussawi J, Vandeputte OM, Monteyne D, Mol A, Pérez‐Morga D, El Jaziri M (2013) A role for the miR396/GRF network in specification of organ type during flower development, as supported by ectopic expression of *Populus trichocarpa* miR396c in transgenic tobacco. Plant Biology **15:** 892-898

Baulcombe D (2004) RNA silencing in plants. Nature **431:** 356-363

Bemer M, Karlova R, Ballester AR, Tikunov YM, Bovy AG, Wolters-Arts M, Rossetto Pde B, Angenent GC, de Maagd RA (2012) The tomato FRUITFULL homologs TDR4/FUL1 and MBP7/FUL2 regulate ethylene-independent aspects of fruit ripening. Plant Cell **24:** 4437-4451

Bergonzi S, Albani MC, Ver Loren van Themaat E, Nordstrom KJ, Wang R, Schneeberger K, Moerland PD, Coupland G (2013) Mechanisms of age-dependent response to winter temperature in perennial flowering of *Arabis alpina*. Science **340:** 1094-1097

Bian H, Xie Y, Guo F, Han N, Ma S, Zeng Z, Wang J, Yang Y, Zhu M (2012) Distinctive expression patterns and roles of the miRNA393/TIR1 homolog module in regulating flag leaf inclination and primary and crown root growth in rice (*Oryza sativa*). New Phytol **196:** 149-161

Boccara M, Sarazin A, Thiebeauld O, Jay F, Voinnet O, Navarro L, Colot V (2014) The Arabidopsis miR472-RDR6 silencing pathway modulates PAMP-and effector-triggered immunity through the post-transcriptional control of disease resistance genes. PLoS Pathogens **10:** e1003883

Bollman KM, Aukerman MJ, Park MY, Hunter C, Berardini TZ, Poethig RS (2003) HASTY, the Arabidopsis ortholog of exportin 5/MSN5, regulates phase change and morphogenesis. Development **130:** 1493-1504

Borsani O, Zhu J, Verslues PE, Sunkar R, Zhu J (2005) Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in Arabidopsis. Cell **123:** 1279-1291

Brodersen P, Sakvarelidze-Achard L, Bruun-Rasmussen M, Dunoyer P, Yamamoto YY, Sieburth L, Voinnet O (2008) Widespread translational inhibition by plant miRNAs and siRNAs. Science **320:** 1185-1190

Casadevall R, Rodriguez RE, Debernardi JM, Palatnik JF, Casati P (2013) Repression of growth regulating factors by the microRNA396 inhibits cell proliferation by UV-B radiation in Arabidopsis leaves. Plant Cell **25:** 3570-3583

Casimiro I, Beeckman T, Graham N, Bhalerao R, Zhang H, Casero P, Sandberg G, Bennett MJ (2003) Dissecting Arabidopsis lateral root development. Trends Plant Sci **8:** 165-171

Chaney R, Ryan J, Li Y, Brown S (1999) Soil cadmium as a threat to human health. Cadmium in soils and plants. Springer, pp 219-256

Chapman EJ, Carrington JC (2007) Specialization and evolution of endogenous small RNA pathways. Nature Reviews Genetics **8:** 884-896

Chen H, Li Z, Xiong L (2012) A plant microRNA regulates the adaptation of roots to drought stress. FEBS Lett **586:** 1742-1747

Chen J, Yang Z, Su Y, Han F, Monts D (2009) Phytoremediation of heavy metal/metalloid-contaminated soils. In: Steinberg RV (Eds), Contaminated Soils: Environmental Impact, Disposal, and Treatment.Nova Science Publishers, Inc. NY, USA.

Chen W, Kong J, Lai T, Manning K, Wu C, Wang Y, Qin C, Li B, Yu Z, Zhang X (2015) Tuning LeSPL-CNR expression by SlymiR157 affects tomato fruit ripening. Scientific Reports **5**

Chen Z, Bao M, Sun Y, Yang Y, Xu X, Wang J, Han N, Bian H, Zhu M (2011) Regulation of auxin response by miR393-targeted transport inhibitor response protein 1 is involved in normal development in Arabidopsis. Plant Mol Biol **77:** 619-629

Chen J, Li WX, Xie D, Peng JR, Ding SW (2004) Viral virulence protein suppresses RNA silencing-mediated defense but upregulates the role of microrna in host gene expression. Plant Cell **16:** 1302-1313

Chiou TJ, Aung K, Lin SI, Wu CC, Chiang SF, Su CL (2006) Regulation of phosphate homeostasis by MicroRNA in Arabidopsis. Plant Cell **18:** 412-421

Chuck G, Candela H, Hake S (2009) Big impacts by small RNAs in plant development. Curr Opin Plant Biol **12:** 81-86

Chuck G, Cigan AM, Saeteurn K, Hake S (2007) The heterochronic maize mutant Corngrass1 results from overexpression of a tandem microRNA. Nat Genet **39:** 544-549

Chung M, Vrebalov J, Alba R, Lee J, McQuinn R, Chung J, Klein P, Giovannoni J (2010) A tomato (*Solanum lycopersicum*) APETALA2/ERF gene, SlAP2a, is a negative regulator of fruit ripening. The Plant Journal **64:** 936-947

Clemens S (2006) Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. Biochimie **88:** 1707-1719

Clive J (2013) Global status of commercialized biotech/GM crops: 2013. ISAAA brief **46:**

Cogoni C, Macino G (2000) Post-transcriptional gene silencing across kingdoms. Curr Opin Genet Dev **10:** 638-643

de Carvalho Niebel F, Frendo P, Van Montagu M, Cornelissen M (1995) Post-transcriptional cosuppression of beta-1,3-glucanase genes does not affect accumulation of transgene nuclear mRNA. Plant Cell **7:** 347-358

De Smet I, Signora L, Beeckman T, Inzé D, Foyer CH, Zhang H (2003) An abscisic acid-sensitive checkpoint in lateral root development of Arabidopsis. The Plant Journal **33:** 543-555

Deak KI, Malamy J (2005) Osmotic regulation of root system architecture. The Plant Journal **43:** 17-28

Debernardi JM, Mecchia MA, Vercruyssen L, Smaczniak C, Kaufmann K, Inze D, Rodriguez RE, Palatnik JF (2014) Post-transcriptional control of GRF transcription factors by microRNA miR396 and GIF co-activator affects leaf size and longevity. The Plant Journal **79:** 413-426

Eamens AL, Agius C, Smith NA, Waterhouse PM, Wang MB (2011) Efficient silencing of endogenous microRNAs using artificial microRNAs in *Arabidopsis thaliana*. Mol Plant **4:** 157-170

Efroni I, Blum E, Goldshmidt A, Eshed Y (2008) A protracted and dynamic maturation schedule underlies Arabidopsis leaf development. Plant Cell **20:** 2293-2306

Fahim M, Millar AA, Wood CC, Larkin PJ (2012) Resistance to Wheat streak mosaic virus generated by expression of an artificial polycistronic microRNA in wheat. Plant Biotechnol J **10:** 150-163

Fang Y, Xie K, Xiong L (2014) Conserved miR164-targeted NAC genes negatively regulate drought resistance in rice. J Exp Bot **65:** 2119-2135

Fei H, Chaillou S, Hirel B, Mahon JD, Vessey KJ (2003) Overexpression of a soybean cytosolic glutamine synthetase gene linked to organ-specific promoters in pea plants grown in different concentrations of nitrate. Planta **216:** 467-474

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature **391:** 806-811

Foley JA, Ramankutty N, Brauman KA, Cassidy ES, Gerber JS, Johnston M, Mueller ND, O'Connell C, Ray DK, West PC, Balzer C, Bennett EM, Carpenter SR, Hill J, Monfreda C, Polasky S, Rockstrom J, Sheehan J, Siebert S, Tilman D, Zaks DP (2011) Solutions for a cultivated planet. Nature **478:** 337-342

Franco-Zorrilla JM, Valli A, Todesco M, Mateos I, Puga MI, Rubio-Somoza I, Leyva A, Weigel D, García JA, Paz-Ares J (2007) Target mimicry provides a new mechanism for regulation of microRNA activity. Nat Genet **39:** 1033-1037

Fu C, Sunkar R, Zhou C, Shen H, Zhang J, Matts J, Wolf J, Mann DG, Stewart CN, Tang Y (2012) Overexpression of miR156 in switchgrass (Panicum virgatum L.) results in various morphological alterations and leads to improved biomass production. Plant Biotechnol J **10:** 443-452

Gao C, Ju Z, Cao D, Zhai B, Qin G, Zhu H, Fu D, Luo Y, Zhu B (2014) MicroRNA profiling analysis throughout tomato fruit development and ripening reveals potential regulatory role of RIN on microRNAs accumulation. Plant Biotechnol J **13**: 370-382

Gasciolli V, Mallory AC, Bartel DP, Vaucheret H (2005) Partially redundant functions of Arabidopsis DICER-like enzymes and a role for DCL4 in producing trans-acting siRNAs. Current Biology **15:** 1494-1500

Gifford ML, Dean A, Gutierrez RA, Coruzzi GM, Birnbaum KD (2008) Cell-specific nitrogen responses mediate developmental plasticity. Proc Natl Acad Sci U S A **105:** 803-808

Godfray HC, Beddington JR, Crute IR, Haddad L, Lawrence D, Muir JF, Pretty J, Robinson S, Thomas SM, Toulmin C (2010) Food security: the challenge of feeding 9 billion people. Science **327:** 812-818

Gonzalez N, De Bodt S, Sulpice R, Jikumaru Y, Chae E, Dhondt S, Van Daele T, De Milde L, Weigel D, Kamiya Y, Stitt M, Beemster GT, Inze D (2010) Increased leaf size: different means to an end. Plant Physiol **153:** 1261-1279

Gregory BD, O'Malley RC, Lister R, Urich MA, Tonti-Filippini J, Chen H, Millar AH, Ecker JR (2008) A link between RNA metabolism and silencing affecting Arabidopsis development. Developmental cell **14:** 854-866

Grigg SP, Canales C, Hay A, Tsiantis M (2005) SERRATE coordinates shoot meristem function and leaf axial patterning in Arabidopsis. Nature **437:** 1022-1026

Gruber BD, Giehl RF, Friedel S, von Wiren N (2013) Plasticity of the Arabidopsis root system under nutrient deficiencies. Plant Physiol **163:** 161-179

Guan Q, Lu X, Zeng H, Zhang Y, Zhu J (2013) Heat stress induction of miR398 triggers a regulatory loop that is critical for thermotolerance in Arabidopsis. The Plant Journal **74:** 840-851

Guo H, Song X, Wang G, Yang K, Wang Y, Niu L, Chen X, Fang R (2014) Plant-generated artificial small RNAs mediated aphid resistance. PloS one **9:** e97410

Guo HS, Xie Q, Fei JF, Chua NH (2005) MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for arabidopsis lateral root development. Plant Cell **17:** 1376-1386

Hackenberg M, Shi B, Gustafson P, Langridge P (2012) A transgenic transcription factor (TaDREB3) in barley affects the expression of microRNAs and other small non-coding RNAs. PloS one **7:** e42030

Hamilton AJ, Baulcombe DC (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. Science **286:** 950-952

Han MH, Goud S, Song L, Fedoroff N (2004) The Arabidopsis double-stranded RNA-binding protein HYL1 plays a role in microRNA-mediated gene regulation. Proc Natl Acad Sci U S A **101:** 1093-1098

Hectors K, Prinsen E, De Coen W, Jansen MA, Guisez Y (2007) *Arabidopsis thaliana* plants acclimated to low dose rates of ultraviolet B radiation show specific changes in morphology and gene expression in the absence of stress symptoms. New Phytol **175:** 255-270

Huang SQ, Xiang AL, Che LL, Chen S, Li H, Song JB, Yang ZM (2010) A set of miRNAs from *Brassica napus* in response to sulphate deficiency and cadmium stress. Plant Biotechnol J **8:** 887-899

Ishida T, Kurata T, Okada K, Wada T (2008) A genetic regulatory network in the development of trichomes and root hairs. Annu.Rev.Plant Biol. **59:** 365-386

Jagadeeswaran G, Saini A, Sunkar R (2009) Biotic and abiotic stress down-regulate miR398 expression in Arabidopsis. Planta **229:** 1009-1014

Jones-Rhoades MW, Bartel DP (2004) Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Mol Cell **14:** 787-799

Jones-Rhoades MW, Bartel DP, Bartel B (2006) MicroRNAs and their regulatory roles in plants. Annu.Rev.Plant Biol. **57:** 19-53

Kakani VG, Reddy KR, Zhao D, Mohammed AR (2003) Effects of ultraviolet-B radiation on cotton (*Gossypium hirsutum* L.) morphology and anatomy. Ann Bot **91:** 817-826

Kalve S, De Vos D, Beemster GT (2014) Leaf development: a cellular perspective. Front Plant Sci **5:**362

Kantar M, Unver T, Budak H (2010) Regulation of barley miRNAs upon dehydration stress correlated with target gene expression. Functional & integrative genomics **10:** 493-507

Karlova R, Rosin FM, Busscher-Lange J, Parapunova V, Do PT, Fernie AR, Fraser PD, Baxter C, Angenent GC, de Maagd RA (2011) Transcriptome and metabolite profiling show that APETALA2a is a major regulator of tomato fruit ripening. Plant Cell **23:** 923-941

Karlova R, van Haarst JC, Maliepaard C, van de Geest H, Bovy AG, Lammers M, Angenent GC, de Maagd RA (2013) Identification of microRNA targets in tomato fruit development using high-throughput sequencing and degradome analysis. J Exp Bot **64:** 1863-1878

Katiyar-Agarwal S, Gao S, Vivian-Smith A, Jin H (2007) A novel class of bacteria-induced small RNAs in Arabidopsis. Genes Dev **21:** 3123-3134

Katiyar-Agarwal S, Morgan R, Dahlbeck D, Borsani O, Villegas A,Jr, Zhu JK, Staskawicz BJ, Jin H (2006) A pathogen-inducible endogenous siRNA in plant immunity. Proc Natl Acad Sci U S A **103:** 18002-18007

Kawashima CG, Matthewman CA, Huang S, Lee B, Yoshimoto N, Koprivova A, Rubio-Somoza I, Todesco M, Rathjen T, Saito K (2011) Interplay of SLIM1 and miR395 in the regulation of sulfate assimilation in Arabidopsis. The Plant Journal **66:** 863-876

Kawashima CG, Yoshimoto N, Maruyama-Nakashita A, Tsuchiya YN, Saito K, Takahashi H, Dalmay T (2009) Sulphur starvation induces the expression of microRNA-395 and one of its target genes but in different cell types. The Plant Journal **57:** 313-321

Kettles GJ, Drurey C, Schoonbeek H, Maule AJ, Hogenhout SA (2013) Resistance of *Arabidopsis thaliana* to the green peach aphid, *Myzus persicae*, involves camalexin and is regulated by microRNAs. New Phytol **198:** 1178-1190

Khan NA, Singh S, Umar S (2008) Sulfur assimilation and abiotic stress in plants. Springer-Verlag, Berlin Heidelberg, Germany 372p

Kim D, Doyle MR, Sung S, Amasino RM (2009a) Vernalization: winter and the timing of flowering in plants. Annu Rev Cell Dev Biol **25:** 277-299

Kim JH, Choi D, Kende H (2003) The AtGRF family of putative transcription factors is involved in leaf and cotyledon growth in Arabidopsis. The Plant Journal **36:** 94-104

Kim JH, Kende H (2004) A transcriptional coactivator, AtGIF1, is involved in regulating leaf growth and morphology in Arabidopsis. Proc Natl Acad Sci U S A **101:** 13374-13379

Kim JH, Woo HR, Kim J, Lim PO, Lee IC, Choi SH, Hwang D, Nam HG (2009b) Trifurcate feed-forward regulation of age-dependent cell death involving miR164 in Arabidopsis. Science **323:** 1053-1057

Knauf VC (1987) The application of genetic engineering to oilseed crops. Trends Biotechnol **5:** 40-47

Koyama T, Furutani M, Tasaka M, Ohme-Takagi M (2007) TCP transcription factors control the morphology of shoot lateral organs via negative regulation of the expression of boundary-specific genes in Arabidopsis. Plant Cell **19:** 473-484

Koyama T, Mitsuda N, Seki M, Shinozaki K, Ohme-Takagi M (2010) TCP transcription factors regulate the activities of ASYMMETRIC LEAVES1 and miR164, as well as the auxin response, during differentiation of leaves in Arabidopsis. Plant Cell **22:** 3574-3588

KUNG Y, LIN S, HUANG Y, CHEN T, Harish SS, CHUA N, YEH S (2012) Multiple artificial microRNAs targeting conserved motifs of the replicase gene confer robust transgenic resistance to negative-sense single-stranded RNA plant virus. Mol Plant Pathol **13:** 303-317

Kurihara Y, Takashi Y, Watanabe Y (2006) The interaction between DCL1 and HYL1 is important for efficient and precise processing of pri-miRNA in plant microRNA biogenesis. RNA **12:** 206-212

Kurihara Y, Watanabe Y (2004) Arabidopsis micro-RNA biogenesis through Dicer-like 1 protein functions. Proc Natl Acad Sci U S A **101:** 12753-12758

Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T (2001) Identification of novel genes coding for small expressed RNAs. Science **294:** 853-858

Lau NC, Lim LP, Weinstein EG, Bartel DP (2001) An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. Science **294:** 858-862

Laubinger S, Sachsenberg T, Zeller G, Busch W, Lohmann JU, Ratsch G, Weigel D (2008) Dual roles of the nuclear cap-binding complex and SERRATE in pre-mRNA splicing and microRNA processing in *Arabidopsis thaliana.* Proc Natl Acad Sci U S A **105:** 8795-8800

Laufs P, Peaucelle A, Morin H, Traas J (2004) MicroRNA regulation of the CUC genes is required for boundary size control in Arabidopsis meristems. Development **131:** 4311-4322

Lee RC, Feinbaum RL, Ambros V (1993) The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell **75:** 843-854

Lee RC, Ambros V (2001) An extensive class of small RNAs in *Caenorhabditis elegans*. Science **294:** 862-864

Li S, Liu L, Zhuang X, Yu Y, Liu X, Cui X, Ji L, Pan Z, Cao X, Mo B (2013) MicroRNAs inhibit the translation of target mRNAs on the endoplasmic reticulum in Arabidopsis. Cell **153:** 562-574

Li X, Wang X, Zhang S, Liu D, Duan Y, Dong W (2012a) Identification of soybean microRNAs involved in soybean cyst nematode infection by deep sequencing. PLoS One **7:** e39650

Li Y, Zheng Y, Addo-Quaye C, Zhang L, Saini A, Jagadeeswaran G, Axtell MJ, Zhang W, Sunkar R (2010) Transcriptome-wide identification of microRNA targets in rice. The Plant Journal **62:** 742-759

Li J, Guo G, Guo W, Guo G, Tong D, Ni Z, Sun Q, Yao Y (2012b) miRNA164-directed cleavage of ZmNAC1 confers lateral root development in maize (Zea mays L.). BMC Plant Biol **12:** 220-2229-12-220

Liang G, Yang F, Yu D (2010) MicroRNA395 mediates regulation of sulfate accumulation and allocation in *Arabidopsis thaliana.* The Plant Journal **62:** 1046-1057

Liu D, Song Y, Chen Z, Yu D (2009b) Ectopic expression of miR396 suppresses GRF target gene expression and alters leaf growth in Arabidopsis. Physiol Plantarum **136:** 223-236

Liu H, Jia S, Shen D, Liu J, Li J, Zhao H, Han S, Wang Y (2012) Four AUXIN RESPONSE FACTOR genes downregulated by microRNA167 are associated with growth and development in *Oryza sativa*. Functional Plant Biology **39:** 736-744

Liu Q, Chen Y (2009) Insights into the mechanism of plant development: interactions of miRNAs pathway with phytohormone response. Biochem Biophys Res Commun **384:** 1-5

Liu H, Guo S, Xu Y, Li C, Zhang Z, Zhang D, Xu S, Zhang C, Chong K (2014) OsmiR396d-regulated OsGRFs function in floral organogenesis in rice through binding to their targets OsJMJ706 and OsCR4. Plant Physiol **165:** 160-174

Liu HH, Tian X, Li YJ, Wu CA, Zheng CC (2008) Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*. RNA **14:** 836-843

Llave C (2004) MicroRNAs: more than a role in plant development? Mol Plant Pathol **5:** 361-366

Llave C, Kasschau KD, Rector MA, Carrington JC (2002) Endogenous and silencing-associated small RNAs in plants. Plant Cell **14:** 1605-1619

Lobbes D, Rallapalli G, Schmidt DD, Martin C, Clarke J (2006) SERRATE: a new player on the plant microRNA scene. EMBO Rep **7:** 1052-1058

Lu X, Guan Q, Zhu J (2013) Downregulation of CSD2 by a heat-inducible miR398 is required for thermotolerance in Arabidopsis. Plant Signal Behav **8:** e24952

Lu C, Jeong DH, Kulkarni K, Pillay M, Nobuta K, German R, Thatcher SR, Maher C, Zhang L, Ware D, Liu B, Cao X, Meyers BC, Green PJ (2008) Genome-wide analysis for discovery of rice microRNAs reveals natural antisense microRNAs (nat-miRNAs). Proc Natl Acad Sci U S A **105:** 4951-4956

Lu S, Sun YH, Shi R, Clark C, Li L, Chiang VL (2005) Novel and mechanical stress-responsive MicroRNAs in Populus trichocarpa that are absent from Arabidopsis. Plant Cell **17:** 2186-2203

Lucca P, Poletti S, Sautter C (2006) Genetic engineering approaches to enrich rice with iron and vitamin A. Physiol Plantarum **126:** 291-303

Luo Y, Guo Z, Li L (2013) Evolutionary conservation of microRNA regulatory programs in plant flower development. Dev Biol **380:** 133-144

Malamy J (2005) Intrinsic and environmental response pathways that regulate root system architecture. Plant, Cell Environ **28:** 67-77

Mallory AC, Dugas DV, Bartel DP, Bartel B (2004) MicroRNA regulation of NAC-domain targets is required for proper formation and separation of adjacent embryonic, vegetative, and floral organs. Current Biology **14:** 1035-1046

Mallory AC, Reinhart BJ, Bartel D, Vance VB, Bowman LH (2002) A viral suppressor of RNA silencing differentially regulates the accumulation of short interfering RNAs and micro-RNAs in tobacco. Proc Natl Acad Sci U S A **99:** 15228-15233

Manning K, Tör M, Poole M, Hong Y, Thompson AJ, King GJ, Giovannoni JJ, Seymour GB (2006) A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. Nat Genet **38:** 948-952

Marker C, Zemann A, Terhörst T, Kiefmann M, Kastenmayer JP, Green P, Bachellerie J, Brosius J, Hüttenhofer A (2002) Experimental RNomics: identification of 140 candidates for small non-messenger RNAs in the plant *Arabidopsis thaliana*. Current Biology **12:**

Meng Y, Ma X, Chen D, Wu P, Chen M (2010) MicroRNA-mediated signaling involved in plant root development. Biochem Biophys Res Commun **393:** 345-349

Miura K, Ikeda M, Matsubara A, Song X, Ito M, Asano K, Matsuoka M, Kitano H, Ashikari M (2010) OsSPL14 promotes panicle branching and higher grain productivity in rice. Nat Genet **42:** 545-549

Morris ER, Chevalier D, Walker JC (2006) DAWDLE, a forkhead-associated domain gene, regulates multiple aspects of plant development. Plant Physiol **141:** 932-941

Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. Plant Cell **2:** 279-289

Naqvi AR, Haq Q, Mukherjee SK (2010) MicroRNA profiling of tomato leaf curl new delhi virus (tolcndv) infected tomato leaves indicates that deregulation of mir159/319 and mir172 might be linked with leaf curl disease. Virol J **7:** 281

Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, Voinnet O, Jones JD (2006) A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science **312:** 436-439

Navarro L, Jay F, Nomura K, He SY, Voinnet O (2008) Suppression of the microRNA pathway by bacterial effector proteins. Science **321:** 964-967

Ori N, Cohen AR, Etzioni A, Brand A, Yanai O, Shleizer S, Menda N, Amsellem Z, Efroni I, Pekker I (2007) Regulation of LANCEOLATE by miR319 is required for compound-leaf development in tomato. Nat Genet **39:** 787-791

Pacheco R, García-Marcos A, Barajas D, Martiáñez J, Tenllado F (2012) PVX–potyvirus synergistic infections differentially alter microRNA accumulation in *Nicotiana benthamiana*. Virus Res **165:** 231-235

Palatnik JF, Allen E, Wu X, Schommer C, Schwab R, Carrington JC, Weigel D (2003) Control of leaf morphogenesis by microRNAs. Nature **425:** 257-263

Palatnik JF, Wollmann H, Schommer C, Schwab R, Boisbouvier J, Rodriguez R, Warthmann N, Allen E, Dezulian T, Huson D (2007) Sequence and expression differences underlie functional specialization of Arabidopsis microRNAs miR159 and miR319. Dev Cell **13:** 115-125

Pandey SP, Baldwin IT (2007) RNA-directed RNA polymerase 1 (RdR1) mediates the resistance of *Nicotiana attenuata* to herbivore attack in nature. The Plant Journal **50:** 40-53

Pandey SP, Shahi P, Gase K, Baldwin IT (2008) Herbivory-induced changes in the small-RNA transcriptome and phytohormone signaling in *Nicotiana attenuata*. Proc Natl Acad Sci U S A **105:** 4559-4564

Park W, Li J, Song R, Messing J, Chen X (2002) CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana*. Current Biology **12:** 1484-1495

Parry G, Calderon-Villalobos LI, Prigge M, Peret B, Dharmasiri S, Itoh H, Lechner E, Gray WM, Bennett M, Estelle M (2009) Complex regulation of the TIR1/AFB family of auxin receptors. Proc Natl Acad Sci U S A **106:** 22540-22545

Pattanaik S, Patra B, Singh SK, Yuan L (2014) An overview of the gene regulatory network controlling trichome development in the model plant, Arabidopsis. Frontiers in plant science **5:**

Peng T, Sun H, Du Y, Zhang J, Li J, Liu Y, Zhao Y, Zhao Q (2013) Characterization and expression patterns of microRNAs involved in rice grain filling. PloS one **8:** e54148

Peng T, Sun H, Qiao M, Zhao Y, Du Y, Zhang J, Li J, Tang G, Zhao Q (2014) Differentially expressed microRNA cohorts in seed development may contribute to poor grain filling of inferior spikelets in rice. BMC Plant Biol **14:** 196

Perez-Torres CA, Lopez-Bucio J, Cruz-Ramirez A, Ibarra-Laclette E, Dharmasiri S, Estelle M, Herrera-Estrella L (2008) Phosphate availability alters lateral root development in Arabidopsis by modulating auxin sensitivity via a mechanism involving the TIR1 auxin receptor. Plant Cell **20:** 3258-3272

Phalan B, Balmford A, Green RE, Scharlemann JPW (2011) Minimising the harm to biodiversity of producing more food globally. Food Policy **36, Supplement 1:** S62-S71

Pradhan B, Naqvi AR, Saraf S, Mukherjee SK, Dey N (2015) Prediction and characterization of Tomato leaf curl New Delhi virus (ToLCNDV) responsive novel microRNAs in *Solanum lycopersicum*. Virus Res **195:** 183-195

Privalle LS, Chen J, Clapper G, Hunst P, Spiegelhalter F, Zhong CX (2012) Development of an agricultural biotechnology crop product: testing from discovery to commercialization. J Agric Food Chem **60:** 10179-10187

Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B, Bartel DP (2002) MicroRNAs in plants. Genes Dev **16:** 1616-1626

Reyes JL, Chua N (2007) ABA induction of miR159 controls transcript levels of two MYB factors during Arabidopsis seed germination. The Plant Journal **49:** 592-606

Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP (2002) Prediction of plant microRNA targets. Cell **110:** 513-520

Robson TM, Aphalo PJ (2012) Species-specific effect of UV-B radiation on the temporal pattern of leaf growth. Physiol Plantarum **144:** 146-160

Rodriguez RE, Mecchia MA, Debernardi JM, Schommer C, Weigel D, Palatnik JF (2010) Control of cell proliferation in *Arabidopsis thaliana* by microRNA miR396. Development **137:** 103-112

Schmitz RJ, Hong L, Fitzpatrick KE, Amasino RM (2007) DICER-LIKE 1 and DICER-LIKE 3 redundantly act to promote flowering via repression of FLOWERING LOCUS C in *Arabidopsis thaliana.* Genetics **176:** 1359-1362

Schommer C, Palatnik JF, Aggarwal P, Chételat A, Cubas P, Farmer EE, Nath U, Weigel D (2008) Control of jasmonate biosynthesis and senescence by miR319 targets. PLoS biology **6:** e230

Schommer C, Debernardi JM, Bresso EG, Rodriguez RE, Palatnik JF (2014) Repression of cell proliferation by miR319-regulated TCP4. Mol Plant **7:** 1533-1544

Schwab R, Palatnik JF, Riester M, Schommer C, Schmid M, Weigel D (2005) Specific effects of microRNAs on the plant transcriptome. Dev cell **8:** 517-527

Seo J, Wu J, Lii Y, Li Y, Jin H (2013) Contribution of small RNA pathway components in plant immunity. Mol Plant-Microbe Interact **26:** 617-625

Serna L, Martin C (2006) Trichomes: different regulatory networks lead to convergent structures. Trends Plant Sci **11:** 274-280

Sun G (2012) MicroRNAs and their diverse functions in plants. Plant Mol Biol **80:** 17-36

Sunkar R, Zhu J (2007) Micro RNAs and short-interfering RNAs in plants. Journal of Integrative Plant Biology **49:** 817-826

Sunkar R, Girke T, Jain PK, Zhu JK (2005) Cloning and characterization of microRNAs from rice. Plant Cell **17:** 1397-1411

Sunkar R, Kapoor A, Zhu JK (2006) Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in Arabidopsis is mediated by downregulation of miR398 and important for oxidative stress tolerance. Plant Cell **18:** 2051-2065

Thiebaut F, Rojas CA, Almeida KL, Grativol C, Domiciano GC, Lamb CRC, DE ALMEIDA ENGLER J, Hemerly AS, Ferreira PC (2012) Regulation of miR319 during cold stress in sugarcane. Plant, Cell Environ **35:** 502-512

Tigchelaar E, Tomes M, Kerr E, Barman R (1973) A new fruit ripening mutant, non-ripening (nor). Rep Tomato Genet Coop **23:** 33

Tilman D, Balzer C, Hill J, Befort BL (2011) Global food demand and the sustainable intensification of agriculture. Proc Natl Acad Sci U S A **108:** 20260-20264

Tscharntke T, Clough Y, Wanger TC, Jackson L, Motzke I, Perfecto I, Vandermeer J, Whitbread A (2012) Global food security, biodiversity conservation and the future of agricultural intensification. Biol Conserv **151:** 53-59

van der Krol AR, Mur LA, Beld M, Mol JN, Stuitje AR (1990) Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell **2:** 291-299

van der Weele CM, Spollen WG, Sharp RE, Baskin TI (2000) Growth of Arabidopsis thaliana seedlings under water deficit studied by control of water potential in nutrient-agar media. J Exp Bot **51:** 1555-1562

Vaucheret H (2006) Post-transcriptional small RNA pathways in plants: mechanisms and regulations. Genes Dev **20:** 759-771

Vazquez F, Gasciolli V, Crété P, Vaucheret H (2004) The nuclear dsRNA binding protein HYL1 is required for microRNA accumulation and plant development, but not posttranscriptional transgene silencing. Current Biology **14:** 346-351

Vidal EA, Araus V, Lu C, Parry G, Green PJ, Coruzzi GM, Gutierrez RA (2010) Nitrate-responsive miR393/AFB3 regulatory module controls root system architecture in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A **107:** 4477-4482

Voinnet O (2009) Origin, biogenesis, and activity of plant microRNAs. Cell **136:** 669-687

Vrebalov J, Ruezinsky D, Padmanabhan V, White R, Medrano D, Drake R, Schuch W, Giovannoni J (2002) A MADS-box gene necessary for fruit ripening at the tomato ripening-inhibitor (rin) locus. Science **296:** 343-346

Wagner GJ, Wang E, Shepherd RW (2004) New approaches for studying and exploiting an old protuberance, the plant trichome. Ann Bot **93:** 3-11

Wahl V, Ponnu J, Schlereth A, Arrivault S, Langenecker T, Franke A, Feil R, Lunn JE, Stitt M, Schmid M (2013) Regulation of flowering by trehalose-6-phosphate signaling in *Arabidopsis thaliana.* Science **339:** 704-707

Wang C, Zhang S, Yu Y, Luo Y, Liu Q, Ju C, Zhang Y, Qu L, Lucas WJ, Wang X (2014a) MiR397b regulates both lignin content and seed number in Arabidopsis via modulating a laccase involved in lignin biosynthesis. Plant Biotechnol J **12:** 1132-1142

Wang S, Wu K, Yuan Q, Liu X, Liu Z, Lin X, Zeng R, Zhu H, Dong G, Qian Q (2012a) Control of grain size, shape and quality by OsSPL16 in rice. Nat Genet **44:** 950-954

Wang S, Chen J (2014) Regulation of cell fate determination by single-repeat R3 MYB transcription factors in Arabidopsis. Frontiers in plant science **5:**133

Wang S, Sun X, Hoshino Y, Yu Y, Jia B, Sun Z, Sun M, Duan X, Zhu Y (2014b) MicroRNA319 positively regulates cold tolerance by targeting OsPCF6 and OsTCP21 in rice (Oryza sativa L.). PloS one **9:** e91357

Wang W, Vinocur B, Altman A (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta **218:** 1-14

Wang Y, Sun F, Cao H, Peng H, Ni Z, Sun Q, Yao Y (2012b) TamiR159 directed wheat TaGAMYB cleavage and its involvement in anther development and heat response. PloS one **7:** e48445

Wang JW (2014) Regulation of flowering time by the miR156-mediated age pathway. J Exp Bot **65:** 4723-4730

Wargent JJ, Moore JP, Roland Ennos A, Paul ND (2009) Ultraviolet radiation as a limiting factor in leaf expansion and development. Photochem Photobiol **85:** 279-286

Wesley SV, Helliwell CA, Smith NA, Wang M, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. The Plant Journal **27:** 581-590

Wu L, Zhang D, Xue M, Qian J, He Y, Wang S (2014) Overexpression of the maize GRF10, an endogenous truncated growth-regulating factor protein, leads to reduction in leaf size and plant height. Journal of integrative plant biology **56:** 1053-1063

Xia K, Wang R, Ou X, Fang Z, Tian C, Duan J, Wang Y, Zhang M (2012) OsTIR1 and OsAFB2 downregulation via OsmiR393 overexpression leads to more tillers, early flowering and less tolerance to salt and drought in rice. PLoS One **7:** e30039

Xie Z, Kasschau KD, Carrington JC (2003) Negative feedback regulation of Dicer-Like1 in Arabidopsis by microRNA-guided mRNA degradation. Current Biology **13:** 784-789

Xie K, Shen J, Hou X, Yao J, Li X, Xiao J, Xiong L (2012) Gradual increase of miR156 regulates temporal expression changes of numerous genes during leaf development in rice. Plant Physiol **158:** 1382-1394

Xie Q, Frugis G, Colgan D, Chua NH (2000) Arabidopsis NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development. Genes Dev **14:** 3024-3036

Xie Z, Allen E, Fahlgren N, Calamar A, Givan SA, Carrington JC (2005) Expression of Arabidopsis MIRNA genes. Plant Physiol **138:** 2145-2154

Xiong L, Wang RG, Mao G, Koczan JM (2006) Identification of drought tolerance determinants by genetic analysis of root response to drought stress and abscisic Acid. Plant Physiol **142:** 1065-1074

Xue Mei S, Bo L, Si Qi HUANG, Surya Kant MEHTA, Lai Lang X (2007) Coordinated expression of sulfate transporters and its relation with sulfur metabolites in *Brassica napus* exposed to cadmium. Botanical Studies **48:** 43-54

Xue X, Zhao B, Chao L, Chen D, Cui W, Mao Y, Wang L, Chen X (2014) Interaction between Two Timing MicroRNAs Controls Trichome Distribution in Arabidopsis. PLoS genetics **10:** e1004266

Xue LJ, Zhang JJ, Xue HW (2009) Characterization and expression profiles of miRNAs in rice seeds. Nucleic Acids Res **37:** 916-930

Yan Y, Wang H, Hamera S, Chen X, Fang R (2014) miR444a has multiple functions in the rice nitrate-signaling pathway. The Plant Journal **78:** 44-55

Yang C, Ye Z (2013) Trichomes as models for studying plant cell differentiation. Cellular and Molecular Life Sciences **70:** 1937-1948

Yang C, Li D, Mao D, Liu X, Ji C, Li X, Zhao X, Cheng Z, Chen C, Zhu L (2013) Overexpression of microRNA319 impacts leaf morphogenesis and leads to enhanced cold tolerance in rice (*Oryza sativa* L.). Plant, Cell Environ **36:** 2207-2218

Yang L, Liu Z, Lu F, Dong A, Huang H (2006) SERRATE is a novel nuclear regulator in primary microRNA processing in Arabidopsis. The Plant Journal **47:** 841-850

Yang Z (2013) A potential role of microRNAs in plant response to metal toxicity. Metallomics **5:** 1184-1190

Yang Z, Ebright YW, Yu B, Chen X (2006b) HEN1 recognizes 21-24 nt small RNA duplexes and deposits a methyl group onto the 2' OH of the 3' terminal nucleotide. Nucleic Acids Res **34:** 667-675

Yant L, Mathieu J, Dinh TT, Ott F, Lanz C, Wollmann H, Chen X, Schmid M (2010) Orchestration of the floral transition and floral development in Arabidopsis by the bifunctional transcription factor APETALA2. Plant Cell **22:** 2156-2170

Yu N, Cai WJ, Wang S, Shan CM, Wang LJ, Chen XY (2010) Temporal control of trichome distribution by microRNA156-targeted SPL genes in Arabidopsis thaliana. Plant Cell **22:** 2322-2335

Zamore PD, Tuschl T, Sharp PA, Bartel DP (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell **101:** 25-33

Zhang D, Trudeau VL (2008) The XS domain of a plant specific SGS3 protein adopts a unique RNA recognition motif (RRM) fold. Cell Cycle **7:** 2268-2270

Zhang LW, Song JB, Shu XX, Zhang Y, Yang ZM (2013a) miR395 is involved in detoxification of cadmium in *Brassica napus*. J Hazard Mater **250:** 204-211

Zhang X, Li H, Zhang J, Zhang C, Gong P, Ziaf K, Xiao F, Ye Z (2011) Expression of artificial microRNAs in tomato confers efficient and stable virus resistance in a cell-autonomous manner. Transgenic Res **20:** 569-581

Zhang Y, Yu Y, Wang C, Li Z, Liu Q, Xu J, Liao J, Wang X, Qu L, Chen F (2013b) Overexpression of microRNA OsmiR397 improves rice yield by increasing grain size and promoting panicle branching. Nat Biotechnol **31:** 848-852

Zhao M, Ding H, Zhu J, Zhang F, Li W (2011) Involvement of miR169 in the nitrogen-starvation responses in Arabidopsis. New Phytol **190:** 906-915

Zhou M, Luo H (2014) Role of microRNA319 in creeping bentgrass salinity and drought stress response. Plant signaling & behavior **9:** 1375-1391

Zhou CM, Zhang TQ, Wang X, Yu S, Lian H, Tang H, Feng ZY, Zozomova-Lihova J, Wang JW (2013a) Molecular basis of age-dependent vernalization in *Cardamine flexuosa*. Science **340:** 1097-1100

Zhou L, Liu Y, Liu Z, Kong D, Duan M, Luo L (2010) Genome-wide identification and analysis of drought-responsive microRNAs in *Oryza sativa*. J Exp Bot **61:** 4157-4168

Zhou M, Li D, Li Z, Hu Q, Yang C, Zhu L, Luo H (2013b) Constitutive expression of a miR319 gene alters plant development and enhances salt and drought tolerance in transgenic creeping bentgrass. Plant Physiol **161:** 1375-1391

Zhu QH, Upadhyaya NM, Gubler F, Helliwell CA (2009) Over-expression of miR172 causes loss of spikelet determinacy and floral organ abnormalities in rice (Oryza sativa). BMC Plant Biol **9:** 149-2229-9-149

CHAPTER II - CONSTITUTIVE EXPRESSION OF RICE *MICRORNA528* ALTERS PLANT DEVELOPMENT AND ENHANCES TOLERANCE TO SALINITY STRESS AND NITROGEN STARVATION IN CREEPING BENTGRASS

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Abstract

MicroRNA528 (miR528) is a conserved monocot-specific small RNA that has the potential of mediating multiple stress responses. So far, however, experimental functional studies of miR528 are lacking. Here, we report that overexpression of the rice (*Oryza sativa*) miR528 (*Osa-miR528*) in transgenic creeping bentgrass (*Agrostis stolonifera* L.)

alters plant development and improves plant salt stress and nitrogen (N) deficiency tolerance. Morphologically, miR528-overexpressing transgenic plants display shortened internodes, increased tiller number and upright growth. Improved salt stress resistance is associated with increased water retention, cell membrane integrity, chlorophyll content, capacity for maintaining potassium homeostasis, Catalase (CAT) activity, and with reduced Ascorbic Acid Oxidase (AAO) activity; while enhanced tolerance to N deficiency is associated with increased biomass, total nitrogen accumulation and chlorophyll synthesis, Nitrite Reductase (NiR) activity, and with reduced AAO activity. In addition, *AsAAO* and Copper Ion Binding Protein 1 (*AsCBP1)* are identified as two putative targets of miR528 in creeping bentgrass. Both of them respond to salinity and nitrogen starvation and are significantly down-regulated in miR528-overexpressing transgenics. Our data establish a key role of miR528 in modulating plant growth and development and in plant response to salinity and N deficiency, and indicate the potential of manipulating miR528 for improving plant abiotic stress resistance.

2.1 Introduction

Abiotic stresses, especially drought, salt, and nitrogen (N) deficiency, are limiting factors for plant growth, development, and agricultural productivity. To cope with drought and salt stresses, plants have evolved similar strategies of osmotic adjustment (Zhu, 2002; Munns, 2002). Plants have also evolved salinity-specific adjustments against ionic disequilibrium, which encompass excluding salts entry into the plants and compartmentalizing ions into vacuoles or old leaves (Munns, 1993; Flowers and Yeo, 1992; Yeo, 1998). Another worldwide limiting factor for crop yields is N deficiency,

which triggers reduced leaf growth rate and photosynthetic rate (Chapin III, et al., 1988). Due to the sessile nature of plants, abiotic stresses are unavoidable. Therefore, it is critical to develop reliable procedures to genetically modify plants for improved performance under environmental stresses, and thereby enhance agricultural productivity to meet the ever-growing demands in food production.

Genetic engineering plays an increasingly important role in agronomic trait modifications in crop species. Currently, many genes encoding functional proteins, transcription factors, and proteins involved in signaling pathways have been identified as abiotic stress responsive genes (Masclaux-Daubresse, et al., 2010; Shinozaki and Yamaguchi-Shinozaki, 2007; Turan, et al., 2012). Constitutive expression of some of these genes in transgenic plants has been demonstrated to lead to enhanced salt or drought tolerance (Golldack, et al., 2011; Kim, et al., 2013; Li, et al., 2014; Lu, et al., 2013). However, the details of the regulatory network in plant stress response remain elusive. To improve plant performance under N deficiency conditions, substantial efforts have been concentrated on understanding the physiological and molecular processes determining plant N use efficiency (NUE), including those involved in N uptake, assimilation, translocation and remobilization. A number of crop plants have been genetically engineered to alter single functional genes in these pathways with limited success due to post-transcriptional regulation machineries (Ferrario-Mery, et al., 1998; Fraisier, et al., 2000; Pathak, et al., 2008). Abiotic stresses trigger a wide array of plant morphological and physiological responses. Therefore, a comprehensive understanding of plant response to abiotic stresses and the knowledge of how to manipulate multiple genes

concurrently to integrate responses of plants at various levels under abiotic stresses are the keys to genetically improving plants for enhanced performance under adverse environmental conditions.

Since the discovery of microRNAs (miRNAs), the important small regulatory molecules two decades ago, the roles some of their members play in the complex stress response network have been gradually recognized. Recently, an evolutionarily conserved monocot-specific miRNA, miR528 was identified as responding to multiple stresses, including salt, drought, low-temperature, submergence, nitrate starvation, arsenite and arsenate stresses in maize (*Zea mays*), sugarcane (*Saccharum officinarum*), phalaenopsis orchid (*Phalaenopsis*), and rice (Ferreira, et al., 2012; Nischal, et al., 2012; Xu, et al., 2011; Zhang, et al., 2008; An, et al., 2011; Sharma, et al., 2015). In addition, deep sequencing analysis shows that miR528 is responsive to diazotrophic bacteria in maize and might be involved in cytoplasmic male sterility in the rice (Thiebaut, et al., 2014; Yan, et al., 2015). MiR528 in many grass species, such as rice, maize, sugarcane, sorghum (*Sorghum bicolor*), and *Brachypodium distachyon* has been shown to have an identical mature sequence (UGGAAGGGGCAUGCAGAGGAG; (Kozomara and Griffiths-Jones, 2014), suggesting that miR528 in different monocot species may function similarly to regulating target gene activity. Eleven putative targets of miR528 in rice have been predicted using in silico analysis. Some of them are involved in the oxidation-reduction processes (Dai and Zhao, 2011), implying that miR528 may function as a multi-stress integrator. However, all the stress-responsive data of miR528 obtained so far have been

from genome-wide large-scale analyses. The experimental functional study of miR528 is still lacking.

In this study, we analyzed the role of miR528 in regulating plant response to salinity and N starvation, and the underlying physiological and molecular mechanisms using transgenic analysis in an important perennial grass species, creeping bentgrass (*Agrostis stolonifera*). Using stem-loop reverse transcriptase quantitative PCR (RT-qPCR), we first demonstrated that like its counterpart in annual species, miR528 in creeping bentgrass is also responsive to salinity stress, and water and N deficiency. We then generated transgenic creeping bentgrass plants overexpressing rice primary miR528 (pri-miR528) to evaluate the impact of miR528 on plant development and response to environmental stress. Our data indicate that transgenic plants exhibit altered growth and development and enhanced tolerance to salt stress and N deficiency. Improved salt stress resistance is associated with increased water retention, cell membrane integrity, chlorophyll content, capacity for maintaining potassium (K) homeostasis, CAT activity, and with reduced AAO activity. Enhanced tolerance to N starvation is associated with increased biomass, total N accumulation and chlorophyll synthesis, NiR activity, and with reduced AAO activity. Our findings highlight a functional role for the miR528 in modulating ionic equilibrium, NUE and the oxidation-reduction process to mediate plant response to salinity stress and N starvation. Additionally, we also identified two creeping bentgrass miR528 targets, *AsAAO* and *AsCBP1*, which are homologs of rice *AAO* and *CBP1* and implicated in oxidation-reduction process. We found that both *AsAAO* and *AsCBP1* respond to salinity stress and nitrogen starvation and are significantly down-regulated in

miR528-overexpressing transgenic plants. Collectively, our data demonstrate the importance of miR528 in plant development and its key role as a multi-stress integrator controlling plant response to salinity and N starvation, pointing to the potential of manipulating miR528 for improving plant abiotic stress resistance.

2.2 Results

MiR528 responds to salt, drought, and N deficiency stresses

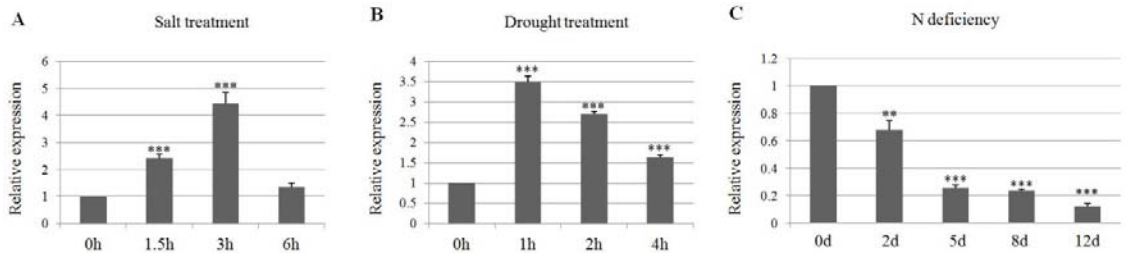


Figure 2.1 Stem-loop RT-qPCR analyses of miR528 expression profiles in response to salt, drought, and N deficiency in WT creeping bentgrass leaves. Relative expression levels of the mature miR528 were determined in WT plants under A, salt treatment, B, drought and C, N deficiency treatment. The relative changes of gene expression were calculated based on the $2^{-\Delta\Delta CT}$ method. *AsUBQ5* was used as an endogenous control. Data are presented as means of three biological replicates x three technical replicates, and error bars represent standard error. A significant difference of expression levels between untreated and each abiotic stress treated WT plants was indicated by asterisks (** or ***) at $P<0.01$, 0.001 by Student's *t-test*.

MiR528 has previously been implicated in plant response to drought, salt, and N deficiency in annual species (Ferreira, et al., 2012; Nischal, et al., 2012; Xu, et al., 2011; Ding, et al., 2009). To study its role in perennial grass species, we first examined its expression in response to various abiotic stresses in creeping bentgrass. Stem-loop RT-qPCR analysis indicates that miR528 is significantly induced by both drought and salt stress, but suppressed under N starvation (Figure 2.1). This suggests that miR528 is

regulated by abiotic stress, consistent with the observations in rice, maize, and sugarcane (Ding, et al., 2009; Ferreira, et al., 2012; Nischal, et al., 2012; Xu, et al., 2011).

Generation and molecular analysis of transgenic creeping bentgrass overexpressing Osa-miR528, a rice miRNA gene

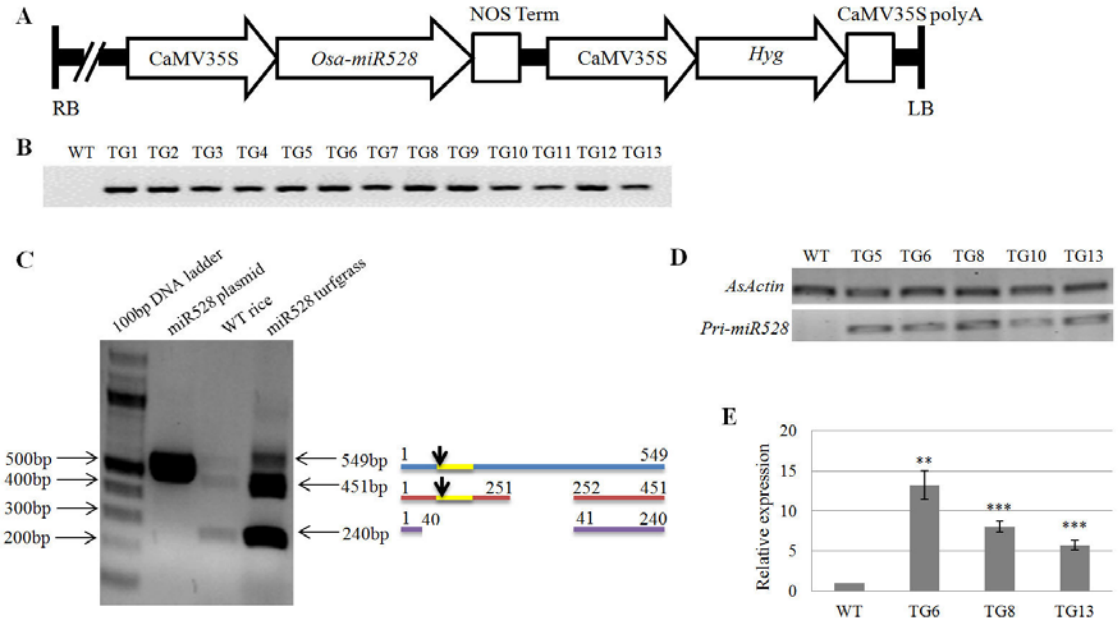


Figure 2.2 Generation and molecular analysis of transgenic (TG) creeping bentgrass overexpressing *Osa-miR528*. A, *Osa-miR528* gene overexpression construct. *Osa-miR528* gene is under the control of Cauliflower Mosaic Virus (CaMV) 35S promoter and linked to the hygromycin resistance gene, *Hyg,* driven by CaMV 35S promoter. RB: right border; LB: left border. B, PCR analysis to amplify *Hyg* gene using genomic DNA of WT and TG creeping bentgrass to determine the integration of *Osa-miR528* gene in the host genome. C, Pri-miR528 shows alternative splicing in WT rice and transgenic creeping bentgrass. The same splicing pattern of the pri-miR528 was observed in both rice and creeping bentgrass. Blue, red, and purple lines represent different splicing patterns. Yellow lines are the locations of pre-miR528. Black arrows indicate the position of mature miR528. D, Additional pair of primers was designed to amplify a common region of the three alternatively spliced pri-miR528 transcripts by using semi-quantitative RT-PCR analysis to compare the expression levels of primary *Osa-miR528* in WT and TG plants. E, Stem-loop RT-qPCR analysis to detect the expression of mature *Osa-miR528* in TG and WT plants. The relative changes in gene expression were calculated based on the $2^{-\Delta\Delta CT}$ method. *AsACT1* was used as an endogenous control. Data are presented as means of three technical replicates, and error bars represent standard error. A significant difference of expression levels between WT and each transgenic line was indicated with asterisks (** or ***) at $P<0.01$ or 0.001 by Student's *t-test*.

To further study the role miR528 plays in plant adaption to abiotic stress, we prepared a miR528 overexpression construct, and introduced it into the genome of WT creeping bentgrass through *Agrobacterium tumefaciens*-mediated transformation. The rice cDNA containing pre-miR528 (Kikuchi, et al., 2003; Satoh, et al., 2007; Liu, et al., 2005) was amplified and then cloned into the binary vector pZH01, generating the *Osa-miR528* overexpression gene construct, p35S-*Osa-miR528*/p35S-*Hyg*. As shown in Figure 2.2A, *Osa-miR528* gene was under the control of the cauliflower mosaic virus (CaMV) 35S promoter and linked to the hygromycin resistance gene, *Hyg*, driven by CaMV 35S promoter. To select positive transgenic plants containing miR528 overexpression constructs, we amplified the *Hyg* gene by PCR grom genomic DNA of regenerated plants and identified a total of 13 transgenic lines (Figure 2.2B), which were morphologically indistinguishable from each other. Three transgenic lines, TG6, TG8 and TG13 were chosen for further characterization on the aspects of plant development and stress response. To verify *Osa-miR528* expression in transgenic plants, we conducted RT-PCR analysis to amplify the cDNA containing the *Osa-miR528* stem-loop structure, comparing the levels of primary *Osa-miR528* transcripts between the WT control and three transgenic lines. Interestingly, besides a fragment with the expected size of 549 bp, two additional bands of 451 bp and 240 bp were also detected (Figure 2.2C). Cloning and sequencing analysis of these DNA fragments revealed that alternative splicing mechanisms are responsible for the production of the additional two bands of 451 bp and 240 bp (data not shown). The same splicing pattern of the pri-miR528 observed in rice (Figure 2.2C) suggesting that molecular mechanisms governing pri-miR528 sequence

processing are conserved in different plant species. An additional pair of primers was then designed to amplify a common region of the three alternatively spliced pri-miR528 transcripts in transgenic lines using semi-quantitative RT-PCR analysis. As demonstrated in Figure 2.2D for the five representative transgenic lines, the transcripts of pri-miR528 are significantly elevated in transgenic lines compared to that in WT controls (Figure 2.2D). To determine whether pri-miR528 can be successfully processed into mature miRNA, we conducted stem-loop RT-qPCR analyses and found that the expression levels of mature *Osa-miR528* in three representative transgenic lines are significantly higher than that in WT control (Figure 2.2E), suggesting that the primary RNA sequence of the *Osa-miR528* from rice is properly processed into mature miRNA in creeping bentgrass.

Overexpression of miR528 alters plant development

To determine the involvement of miR528 in plant development, we analyzed WT and TG plants initiated from a single tiller in pure sand. TG plants produced significantly more, but shorter tillers than WT controls (Figure 2.3, A, C, and J; Figure A-1, A and B), especially at the later developmental stage (10 weeks old; Figure 2.3J). However, no significant difference in the total numbers of shoots, the primary and secondary tillers from a crown and internodes was observed between TG and control plants at the later developmental stages (60 and 90 d old; Figure 2.3K). The developmental changes observed in TG plants were further confirmed by comparing WT and *Osa-miR528* TG plants grown in the same pot filled with soil (Figure 2.3B). In addition, TG plants exhibited more upright tiller growth than WT controls (Figure 2.3B; Figure A-1, C).

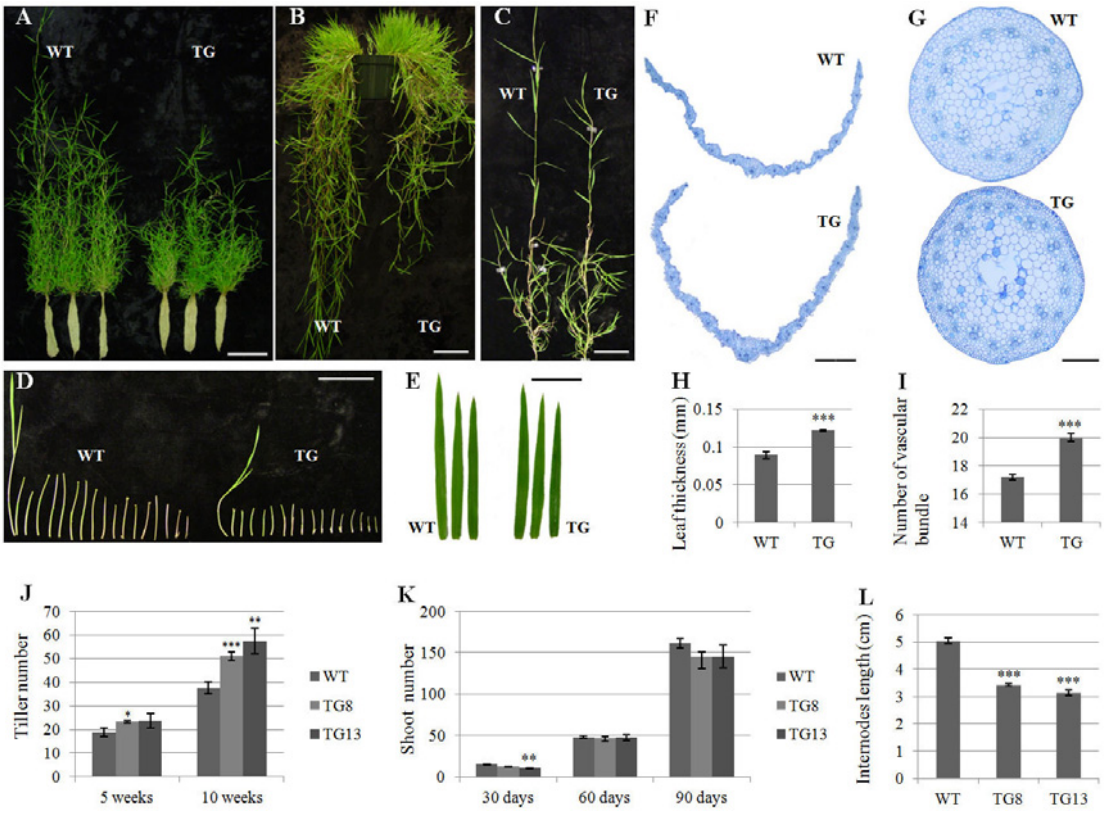


Figure 2.3 Plant tillering and development. A, Ten-week-old WT and TG plants initiated from a single tiller. Scale bar, 10 cm. B, Two-month-old WT and TG plants initiated from the same number of tillers were grown in the same 6-inch pot. Scale bar, 10 cm. C, Close up of the longest tillers from WT and TG plants, respectively. Scale bar, 5 cm. D, All internodes from the representative longest tiller were sliced from top to bottom and arranged from left to right. Scale bar, 5 cm. E, Top three fully developed leaves from the representative tillers of WT and TG plants. Scale bar, 2 cm. F, Cross-section images of WT and TG leaves. Scale bar, 200 µm. G, Cross-section images of WT and TG stems. Scale bar, 100 µm. H, Statistical analysis of leaf thickness between representative WT and TG plants (n=8). I, Statistical analysis of the number of vascular bundles between representative WT and TG stems (n=8). J, Tiller number in WT and TG plants five and ten weeks after initiation from a single tiller (n=5). K, Total shoot number including both tillers and lateral shoots in WT and TG plants at 30, 60, and 90days after initiation from a single tiller (n=5). L, Average length of top eight internodes from WT and TG tillers (n=6). Data are showed as means, and error bars represent standard error. A significant difference between WT and each transgenic line was indicated with asterisks (*, **, or ***) at P<0.05, 0.01, or 0.001 by Student's *t-test*. Tillers are grass shoots growing out from the crown at the base of the plants. Shoots include both tillers and lateral shoots growing out from the tillers.

To further study what causes the reduced tiller length in transgenics, we analyzed the average length and number of the internodes of the representative tillers from WT and TG plants (Figure 2.3D). We found that the total numbers of the internodes in WT and

TG tillers are similar, whereas the average length of the internodes from each tiller in TG plants is significantly reduced compared with WT controls (Figure 2.3L).

TG and WT leaves and stems were also compared at the cellular level via histological analysis (Figure 2.3, E-G). Transgenic leaves were significantly thicker than WT leaves (Figure 2.3H) and the number of the stem vascular bundles was significantly increased in transgenics compared to that in WT controls (Figure 2.3I).

The potential impact of miR528 on plant growth was investigated by measuring the shoot and root biomass of the ten-week-old WT and TG plants initiated from a single tiller, and the weekly clipping weight thereafter for continuous four weeks. Our statistical analyses revealed no significant difference in biomass accumulation between TG and control plants (Figure A-2).

Overexpression of miR528 leads to enhanced salt tolerance in transgenic plants

To investigate the role of miR528 plays in plant stress response, we examined TG and WT plants under salinity stress using fully developed plants (Figure 2.4A). Figure 2.4B and C show plants recovering for eight days after a nine-day exposure to 200 mM NaCl. Salt-elicited tissue damage was significantly more pronounced in WT controls than in TG plants, suggesting an enhanced tolerance to salt stress in miR528-expressing TG plants.

Osa-miR528 transgenics exhibit better water retention, cell membrane integrity, and higher proline and chlorophyll contents than WT controls under salt stress

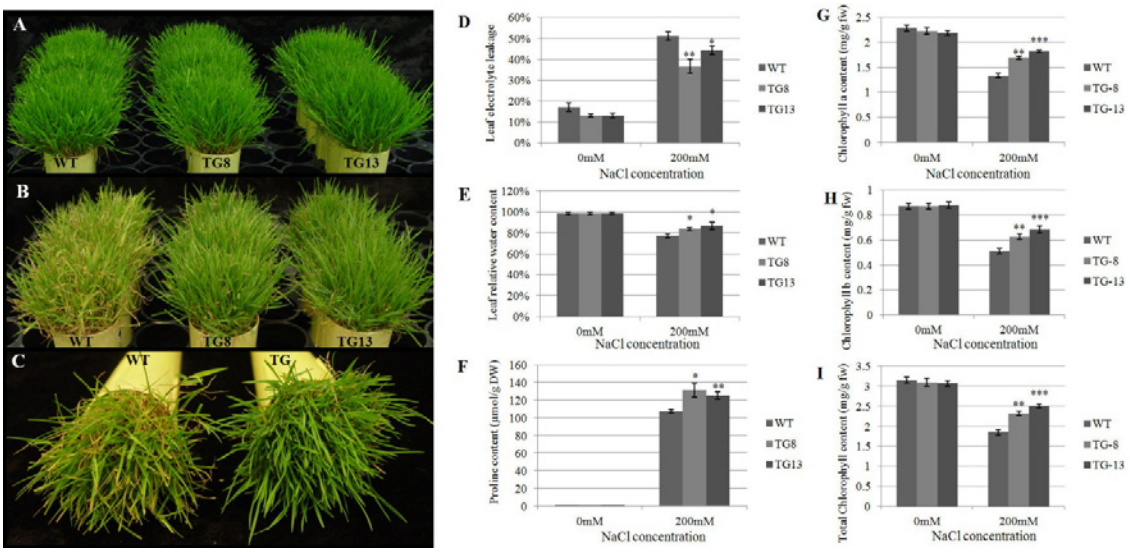


Figure 2.4 Responses of WT controls and transgenics to salinity treatment. A, WT controls and two TG lines initiated from the same amount of tillers were trimmed to the same height before salt stress test. B, Fully developed WT and TG plants were subjected to 200 mM NaCl treatment. Performance of WT and TG plants at the 8 days after recovery from a 9-day salt treatment. C, Close up of representative WT and TG plants from B. D, Electrolyte leakage values were calculated before and after 9-day salt treatment. E, Relative water contents were measured before and after 9-day salt treatment. F, Proline contents of WT and TG leaves were measured before and after 200 mM NaCl treatment. G, Chlorophyll a content, H, Chlorophyll b content, and I, Total chlorophyll content were measured before and after 9-day salt treatment. Data are presented as means (n=5), and error bars represent ±SE. Asterisks (*, ** or ***) indicate a significant difference between WT and transgenic plants at $P<0.05$, 0.01 or 0.001 by Student's *t-test*.

Salinity stress damages plant cell membrane and turgidity. Therefore, the maintenance of cell membrane integrity and water status is considered the major component in plant salt stress tolerance. To investigate the degree of cell membrane injury between WT and TG plants, we measured their electrolyte leakage (EL). Under normal growth conditions, there was no significant difference of EL between WT and two transgenic lines. After a nine-day salt stress treatment, the EL value of WT was

significantly higher than that of TG plants (Figure 2.4D), indicating that TG plants have a better capability of maintaining cell membrane integrity than WT controls under salt stress conditions. To compare the water status in WT and TG plants, we measured the relative water content (RWC). Both transgenics and WT controls displayed similar RWC under normal growth conditions (Figure 2.4E). However, when subjected to salinity stress for nine days, transgenics had significantly higher RWC than WT controls (Figure 2.4E), implying that TG plants have improved ability to retain water under salinity stress.

Proline is essential for plant primary metabolism under salt stress. It functions as a molecular chaperone in buffering the cytosolic redox status within the cell and in ROS scavenging (Ashraf and Foolad, 2007). Proline content in both transgenics and WT controls was similar before salt treatment (Figure 2.4F). However, significantly higher proline accumulated in transgenics than in WT controls after the salt stress (Figure 2.4F), suggesting an enhanced ROS detoxification capacity under osmotic stress in TG plants.

Leaf chlorophyll content is also affected under salt stress due to the destruction of the chlorophyll pigment protein complex, the degradation of the chlorophyll enzyme chlorophyllase, and the interference with the synthesis of chlorophyll structural components (Ali, et al., 2004; Gupta, et al., 1983; Rao and Rao, 1981). In this study, there was no significant difference in chlorophyll content between WT and TG plants under normal growth conditions, whereas all transgenic lines showed significantly higher chlorophyll content than WT controls during salt treatment (Figure 2.4, G-I), suggesting a

possible role the improved photosynthesis of the transgenics plays in contributing to enhanced salt stress resistance.

Osa-miR528 transgenic plants maintain cellular K^+ homeostasis under salt stress

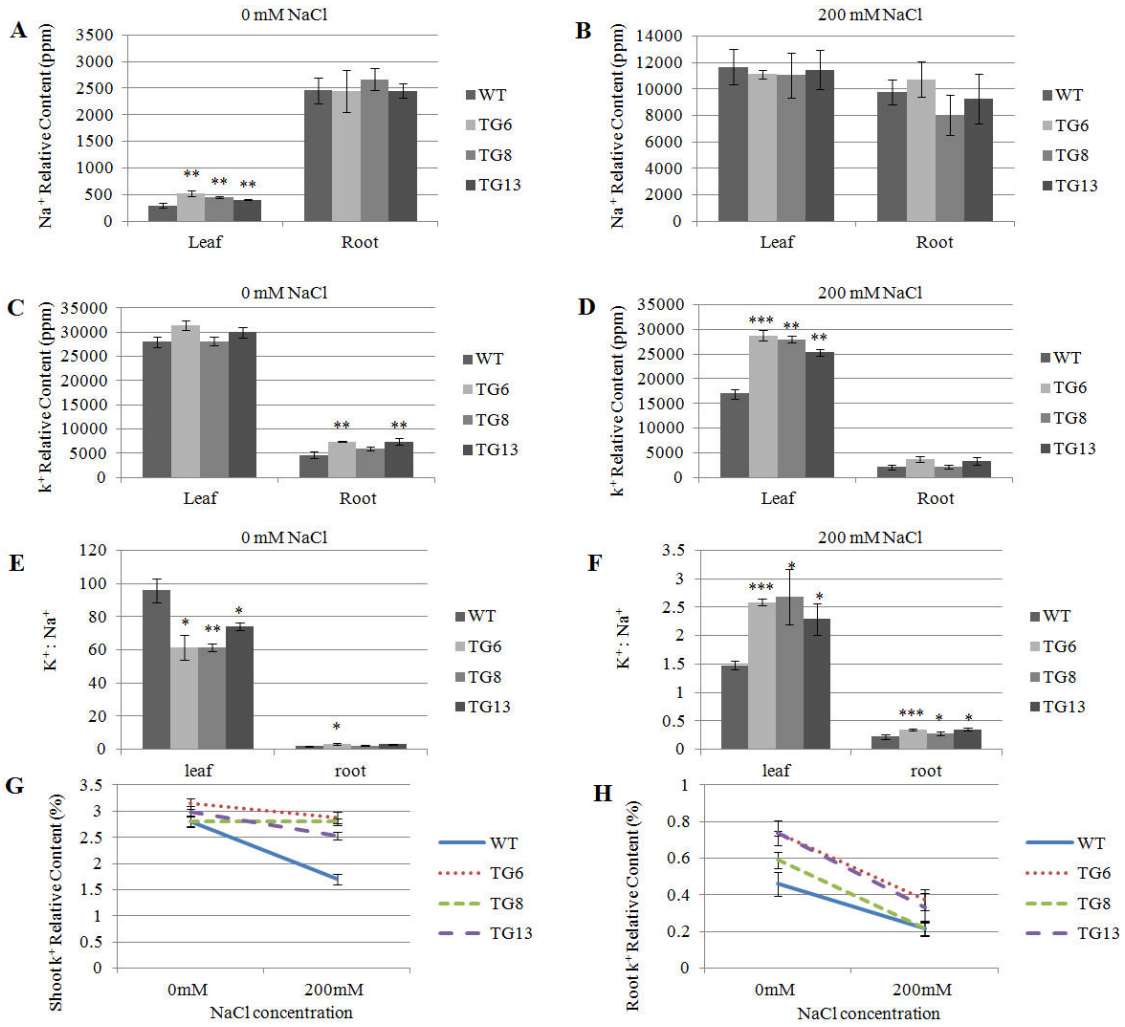


Figure 2.5 Na^+ and K^+ content in WT and TG plants under normal and salt stress conditions. A, Na^+ relative content in shoot and root tissues of WT and TG plants before salinity treatment. B, Na^+ relative content in shoot and root tissues of WT and TG plants 9 days after salinity treatment. C, K^+ relative content in shoot and root tissues of WT and TG plants under normal growth conditions. D, K^+ relative content in shoot and root tissues of WT and TG plants 9 days after salinity treatment. E, K^+: Na^+ ratio in shoots and roots of WT and TG plants before 200 mM NaCl treatment. F, K^+: Na^+ ratio in shoots and roots of WT and TG plants 9 days after salt treatment. G, Shoot K^+ relative content in WT and TG plants before and after salinity stress. H, Root K^+ relative contents in WT and TG plants before and after salinity stress. Data are

presented as means (n=3), and error bars represent standard error. Asterisks (*, **, or ***) indicate significant differences of K^+ content, Na^+ content, or K^+: Na^+ ratio between WT and each transgenic line at $P<0.05$, 0.01, or 0.001 by Student's *t-test*.

Salt stress imposes ionic imbalance and osmotic stress on plants due to elevated Na^+ levels around plant roots. To compare the Na^+ uptake in WT and *Osa-miR528* TG plants, Na^+ relative content was measured. While there was no difference in Na^+ content in roots between transgenics and WT controls, the three transgenic lines accumulated significantly more Na^+ than WT controls in shoots before the salt stress (Figure 2.5A). After the salt treatment, WT and TG plants had similar Na^+ content in both shoots and roots (Figure 2.5B). Potassium (K) plays an essential role in diverse physiological processes including turgor adjustment, stomata movement, cell elongation, and activation of more than 50 cytoplasmic enzymes (Gambale and Uozumi, 2006; Lebaudy, et al., 2007; Marschner, 1995). Salinity also affects K^+ homeostasis, because Na^+ competes with K^+ for binding sites during enzymatic reactions and protein syntheses in the cytoplasm where K^+ functions as a co-factor in these processes (Marschner, 1995). Our result showed that K^+ relative content in WT and TG shoots was similar or slightly higher in TG shoots before salt stress (Figure 2.5C). After salinity treatment, interestingly, transgenics maintained their shoot K^+ level, whereas, the K^+ levels in WT shoots dropped dramatically, becoming significantly lower than that in transgenic shoots (Figure 2.5D). Transgenics also contained higher K^+ in roots than WT plants, although the difference was insignificant (Figure 2.5, C and D). One of the key elements in plant salinity tolerance is the capacity to maintaining a high K^+: Na^+ ratio. Under normal growth conditions, WT shoots had a significantly higher K^+: Na^+ ratio than transgenics due to

their lower Na^+ contents than transgenic shoots (Figure 2.5E). After salt stress treatment, however, K^+: Na^+ ratios of shoots and roots were both significantly higher in transgenics than in WT controls (Figure 2.5F). Figure 5G shows that under salt stress, transgenics were capable of maintaining similar shoot K^+ levels to non-stressed conditions compared to WT controls. However, K^+ levels in both WT and TG roots decreased dramatically although transgenic roots had higher K^+ contents than WT controls under non-stressed conditions (Figure 2.5H).

The impact of miR528 on the expression of the potassium transporter AsHAK5

Differences in Na^+ and K^+ content between WT and TG plants imply that miR528 might mediate the concerted action of ion transport systems. To investigate the underlying mechanism of miR528-mediated ion transport, K transporter genes in creeping bentgrass were identified and their expression was analyzed in TG and WT plants. Previous studies indicate that there are mainly seven gene families involved in K^+ uptake (Maser, et al., 2001; Véry and Sentenac, 2002; Véry and Sentenac, 2003), of which functionally characterized genes encoding K permeable channels and K transporters were selected for further study. *AsHAK5* (KR911825) from the KP/HAK/KT transporter family was successfully amplified in creeping bentgrass and found to be up-regulated in TG leaves and roots compared to WT controls (Figure 2.6), which might contribute to the increased K^+ uptake and enhanced capacity of maintaining K^+ homeostasis in TG plants.

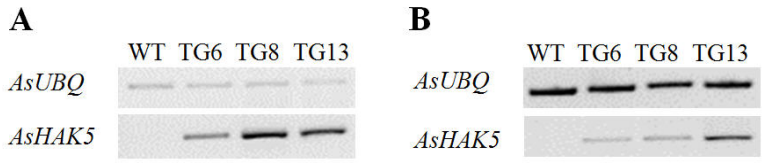


Figure 2.6 Expression levels of *AsHAK5* in WT and TG plants by semi-quantitative RT-PCR analysis. A, Expression levels of *AsHAK5* in leaf tissues of WT and TG plants under normal growth conditions. B, Expression levels of *AsHAK5* in root tissues of WT and TG plants under normal growth conditions. *AsUBQ5* gene was used as the endogenous control.

Overexpression of miR528 results in enhanced ROS scavenging associated with increased CAT activity, but decreased AAO activity under salt stress in transgenics

In addition to the impact on potassium homeostasis, salt stress also causes the accumulation of reactive oxygen species (ROS) in plant cells. Plants have evolved the stress tolerance mechanism of ROS detoxification via increasing antioxidant enzyme activity. Since the predicted targets of *Osa-miR528* contain many antioxidant enzyme-encoding genes, this prompted us to examine how miR528 impacts antioxidant enzyme activity. We first measured the activity of the Cu/Zn superoxide dismutase (SOD) enzyme encoded by a predicted miR528 target gene, but observed no significant difference between WT and TG plants (Figure A-3A). SOD catalyzes the dismutation of superoxide radical into oxygen or hydrogen peroxide; the latter is further decomposed to water and oxygen by CAT. We therefore conducted CAT assay and observed that transgenic plants have significantly higher a CAT activity than WT controls under both normal and salt stress conditions, suggesting an increased capacity of ROS scavenging in miR528 TG plants (Figure A-3B). Another predicted miR528 target encodes AAO, which catalyzes the reaction of ascorbate (AA) oxidation. Under salt stress, transgenic

plants exhibited significantly lower AAO activity than WT controls, indicating that transgenics maintain higher levels of AA under redox status, which contributes to a better elimination of ROS than WT controls (Figure A-3C).

MiR528 transgenics exhibit an enhanced NUE associated with up-regulated gene expression and increased enzyme activity of nitrite reductase

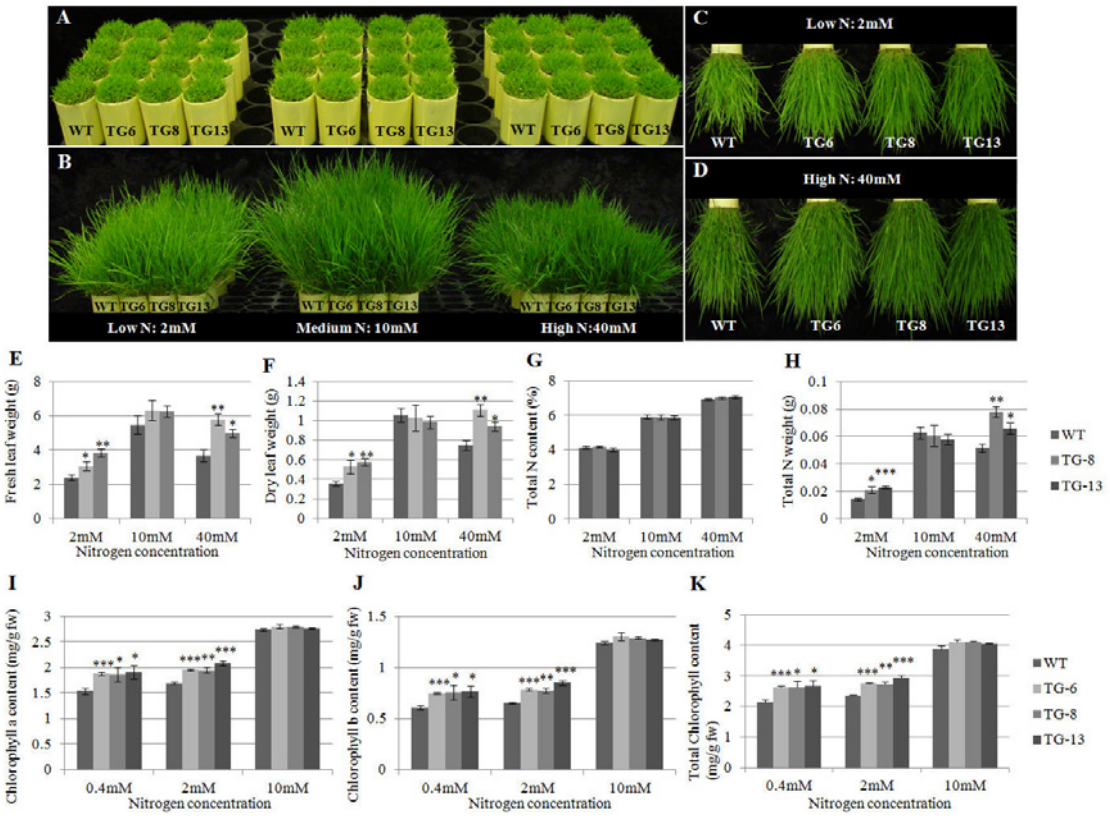


Figure 2.7 Responses of WT and TG plants under different concentrations of N solutions. A, WT and TG plants were trimmed to be uniform before applying N solutions. B, Performance of WT controls and three TG lines on MS solutions containing 2, 10, or 40 mM N for four weeks. C, Close up of WT and TG shoots under 2mM and D, 40mM N solution treatment for four weeks. E, Shoot fresh weight and F, dry weight of WT and TG plants after 4-weeks growth with three different N solutions. G, The percentage of shoot total N content of WT and TG plants was measured four weeks after applying different N solutions. H, The weight of shoot total N was measured four weeks after applying different N solutions. Plant chlorophyll contents including I, Chlorophyll a, J, Chlorophyll b and K, Total chlorophyll was measured four weeks after applying different concentrations of N solutions. Data are presented as means (n=4), and error bars

represent ±SE. Asterisks (*, ** or ***) indicate significant differences of biomass value, total N weight, or chlorophyll contents between WT and transgenic plants at $P<0.05$, 0.01, or 0.001 by Student's *t-test*.

To examine the responses of WT and TG plants under different N supplies (2, 10, and 40 mM), we first determined the optimum N concentration for creeping bentgrass by applying Murashige and Skoog (MS) nutrient solutions containing 2, 10, or 40 mM N respectively to WT and TG plants. Four weeks later, WT and TG plants had the fastest and slowest growth rate with 10 mM and 2 mM N solution treatments, respectively (Figure 2.7B, E and F). Thus, 10 mM is the optimum N concentration in our experiment and was used for further analysis. The result also shows that the excess N level of 40 mM reduces plant growth (Figure 2.7B) which might cause from the decreased uptake of other nutrient elements. When comparing plant growth between transgenics and WT controls, no significant difference in shoot biomass was observed under a normal N supply of 10 mM. However, transgenics grew better and produced significantly more shoot biomass than WT controls under both N deficiency (2 mM) and over-fertilized (40 mM) conditions (Figure 2.7, E and F). Moreover, transgenics also exhibited delayed leaf senescence compared to WT controls. While WT controls exhibited wilting leaf tips under all three treatments of N fertilization, TG plants barely had this symptom, suggesting a role miR528 may play in plant leaf senescence and longevity (Figure A-4).

To further evaluate the impact of miR528 on plant response to N, we measured plant leaf chlorophyll contents in both TG and WT plants under N starvation and normal conditions. In comparison with N-sufficient plants, plants under N deficiency conditions (0.4 mM and 2 mM) had reduced chlorophyll contents, especially under 0.4 mM N

supply (Figure 2.7, I-K). While TG plants were similar to WT controls in chlorophyll content under N-sufficient conditions, they had significantly higher chlorophyll than WT controls under N-starved conditions (Figure 2.7, I-K), suggesting a low degree of chlorophyll degradation, and therefore could be a higher photosynthetic capability in TG plants than in WT controls under N deficiency conditions.

N is an essential nutrient for plant biomass production. To investigate how different amounts of N uptake resulted in distinct plant growth rates, we compared the total N content in WT and TG plants under N-starved (2 mM), N-sufficient (10 mM), and N-excessive (40 mM) conditions. The result indicates that the higher the concentration of the N solution applied was, the more total N plants accumulated (Figure 2.7G). When the total N content was measured as the percentage of the biomass weight, there was no significant difference between WT and TG plants. However, TG shoots accumulated significantly more N than WT controls under N-starved and N-excessive conditions due to their higher shoot biomass under both conditions (Figure 2.7G and H; Figure A-5), suggesting an enhanced NUE in TG plants.

To investigate what causes the enhanced NUE, we examined the transcript levels of the genes encoding key enzymes in N assimilation pathway in WT and transgenic creeping bentgrass plants. The enzymes include nitrate reductase (NR), NiR, glutamine synthetase (GS), and glutamate synthase (GOGAT). The result shows that the expression of *AsNiR* (KR911829) was significantly up-regulated in transgenic plants in comparison with WT controls (Figure 2.8A), but *AsNR* (KR911828), *AsGS* (KR911826), and

AsGOGAT (KR911827) have similar expression levels in WT and TG plants (data not shown).Consistently, the enzyme activity of NiR is also significantly higher in transgenic plants than in WT controls before and after N starvation treatment (Figure 2.8B). It should be noted that NiR activity increased in both WT and TG plants in response to N starvation (Figure 2.8B).

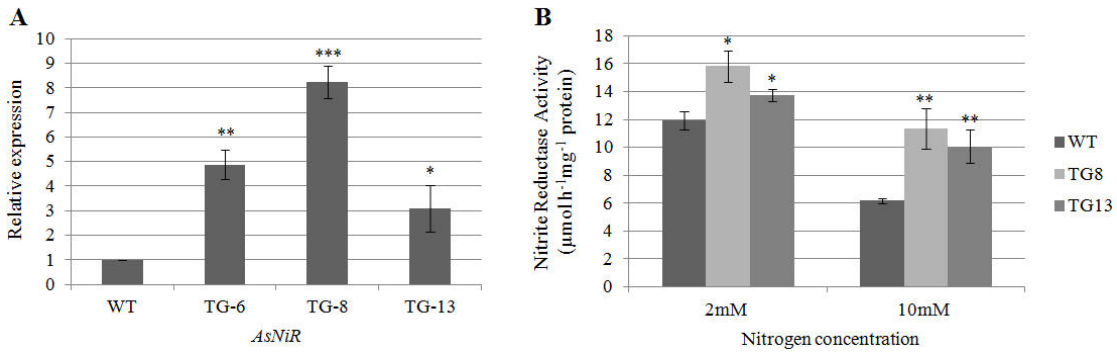
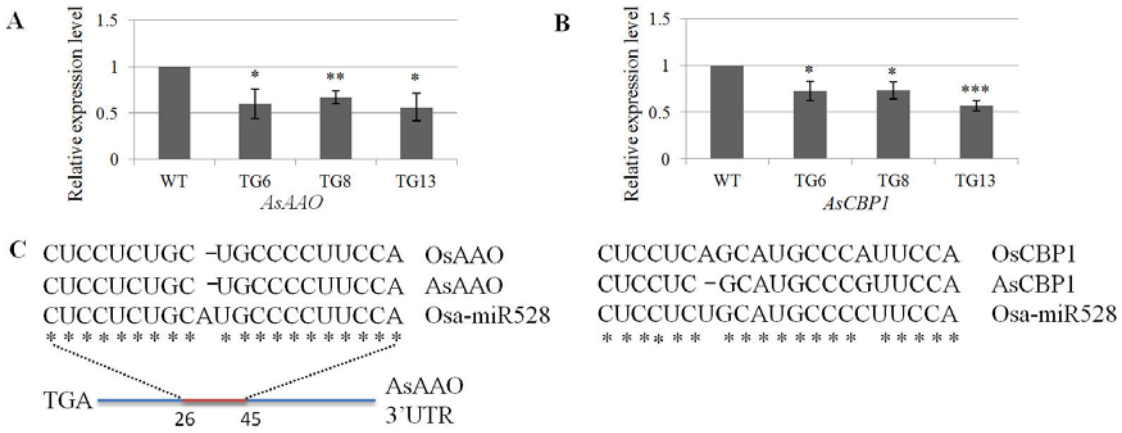


Figure 2.8 AsNiR gene expression analysis and NiR enzyme assays in WT and TG plants. A, RT-qPCR analysis of AsNiR transcript levels in WT plants and three transgenic lines under 10 mM N conditions. AsACT1 was used as an endogenous control. Data are presented as means of three technical replicates and three biological replicates. B, NiR assay in WT controls and two transgenic lines before and two weeks after N starvation. Data are presented as means of three biological replicates. The error bars represent standard error. Asterisks (*, ** or ***) indicate significant differences of expression levels or enzyme activities between WT and TG plants at $P<0.05$, 0.01 or 0.001 by Student's *t-test*.

Overexpression of miR528 leads to decreased AAO activity under N deficiency

N deprivation triggers redox changes and oxidative stress (Kandlbinder, et al., 2004). The predicted miR528 target *AAO* gene and its role in oxidative stress regulation have been discussed above. We therefore conducted plant AAO assays under normal and N starvation conditions. The result indicates that AAO activity in both WT and TG plants increased dramatically when subjected to N starvation, but the increase in TG plants was significantly less pronounced than WT controls (Figure A-6). The lower level of AAO in

transgenics helps maintain relatively high levels of redox AA under N deprivation, and thereby keeping the balance between ROS production and its scavenging under oxidative stress.



D

Putative Targets	Orthologue in rice	Orthologue in *Arabidopsis*	Description	Function
AsAAO	*Os06g37150*	*At5G21100*	L-ascorbate oxidase precursor	Oxidation-reduction
AsCBP1	*Os07g38290*	*At5G26330*	Cupredoxin superfamily protein	Copper ion binding protein

Figure 2.9 Putative *miR528* targets identification in creeping bentgrass. A, Expression levels of *AsAAO* and B, *AsCBP1* in WT and three transgenic lines examined via RT-qPCR. *AsUBQ5* was used as an endogenous control. Data are presented as means of three technical replicates, and error bars represent ±SE. Asterisks (*, ** or ***) indicate a significant difference of expression levels between WT and each transgenic line at P<0.05, 0.01 or 0.001 by Student's t-test. C, A comparison of miR528 target sites in the putative targets *AAO* and *CBP1* between rice and creeping bentgrass. Asterisks indicate the identical RNA sequences. D, Information about the orthologues of the two putative miR528 target genes in rice and Arabidopsis.

Identification of putative Osa-miR528 targets and their responses to stresses

To understand the underlying molecular mechanisms of miR528-mediated plant response to salinity and N deficiency, we sought to identify putative targets of miR528 in creeping bentgrass. Currently, only SsCBP1, a copper ion binding domain-containing

protein is experimentally confirmed as the target of miR528 in sugarcane (Zanca, et al., 2010). In rice, *Os06g37150* encoding AAO has been validated as the target of miR528 through a high-throughput degradome sequencing approach (Wu, et al., 2009b). To identify its targets in creeping bentgrass, a plant small RNA target analysis tool (psRNA Target) was applied to predict targets in rice genome (Dai and Zhao, 2011). Eleven putative targets were recognized in rice, including one copper ion binding protein (Os07g38290), one AAO (Os06g37150), one laccase precursor protein (Os01g44330), one Zn/Cu SOD (Os08g44770), two plastocyanin-like domain containing proteins (Os06g11310 and Os08g04310), one translation initiation factor (Os01g40150), one F-box domain containing protein (Os06g06050), two multicopper oxidase domain containing proteins (Os01g03620 and Os01g03640) and one unknown protein (Os09g33800), of which, partial fragments of four genes were successfully amplified in creeping bentgrass based on the sequence similarity to rice (data not shown). Genes encoding *AAO* and *CBP1* showed decreased expression in TG plants (Figure 2.9, A and B), indicating that they might be targets of miR528 in creeping bentgrass. A MiR528 targeting site in *AsCBP1* (KR911824) was detected in its open reading frame as described in Figure 2.9C. Interestingly, a MiR528 target site was not found in the coding region of *AsAAO*, instead, it was identified by RACE analysis, as located in the 3'UTR from the 26th nt to the 45th nt after the stop codon TGA (Figure 2.9C). The descriptions, functions and corresponding orthologues in rice and Arabidopsis of *AsAAO* and *AsCBP1* are listed in Figure 2.9D. *AsAAO* (KR911823) functions in oxidation-reduction, suggesting its potentially important role in plant abiotic stress response. *AsCBP1* encodes a cupredoxin

superfamily protein. Proteins from this family function in oxidation homeostasis and electron transfer reactions, which are involved in photosynthesis, respiration, cell signaling, and numerous reactions of oxidases and reductases (Dennison, 2005; Lu, et al., 2004; Marshall, et al., 2009; Solomon, et al., 2004).

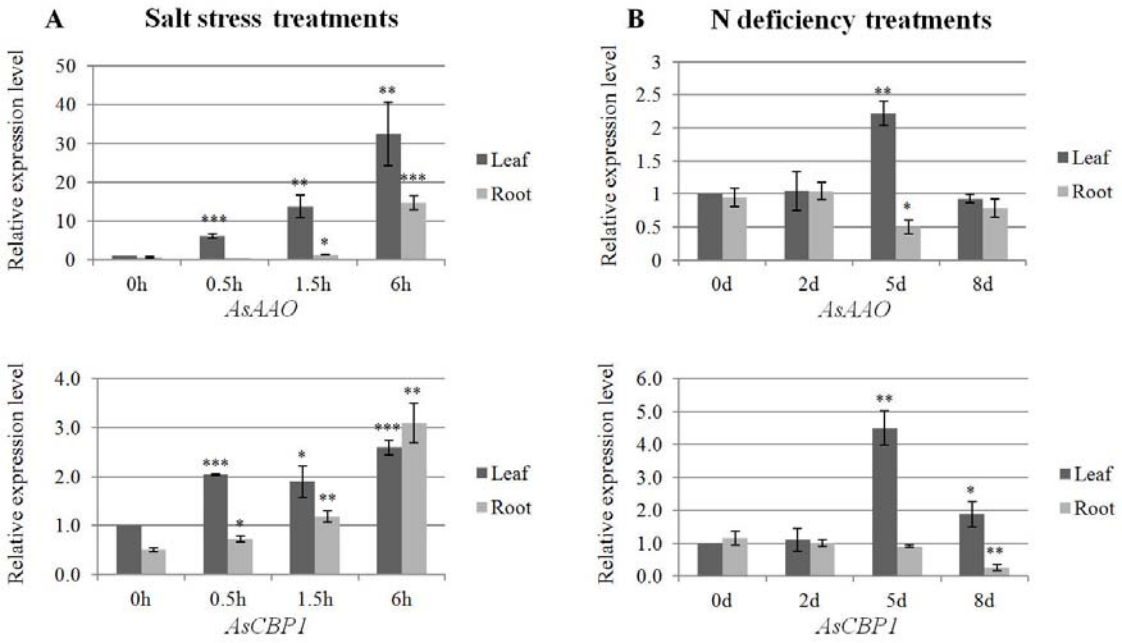


Figure 2.10 Expression patterns of the two *miR528* putative targets under salt and N deficiency conditions through real-time RT-PCR analysis. A, Expression profiles of *AsAAO* and *AsCBP1* in WT leaf and root tissues under 200 mM NaCl treatment (0 to 6 hours). B, Expression profiles of *AsAAO* and *AsCBP1* in WT leaf and root tissues under N starvation (0 mM N) from 0 to 8 days. *AsUBQ5* was used as an endogenous control. Data are presented as means of three technical replicates, and error bars represent standard error. A significant difference of gene expression levels between untreated and stress treated leaf or root tissues was indicated with asterisks (*, ** or ***) at $P<0.05$, 0.01 or 0.001 by Student's *t-test*.

To investigate whether *AsAAO* and *AsCBP1* respond to salt and N deficiency stresses, we conducted real-time RT-PCR analysis to examine their expression profiles under salt and N starvation treatments. Figure 2.10 shows that the expression level of *AsAAO* was significantly induced in response to salt stress in WT leaf and root tissues. When plants

were exposed to N deficiency, the expression of *AsAAO* was significantly induced and repressed five days after treatment in WT leaf and root tissues, respectively (Figure 2.10B). When plants were exposed to salt stress, *AsCBP1* expression in leaf and root tissues was significantly induced (Figure 2.10A). During N starvation, *AsCBP1* was induced five and eight days after treatment in leaf tissues, while its expression declined in root tissues eight days after treatment (Figure 2.10B).

MiR528 has crosstalk with other abiotic stress-related miRNAs and genes in perennial grasses

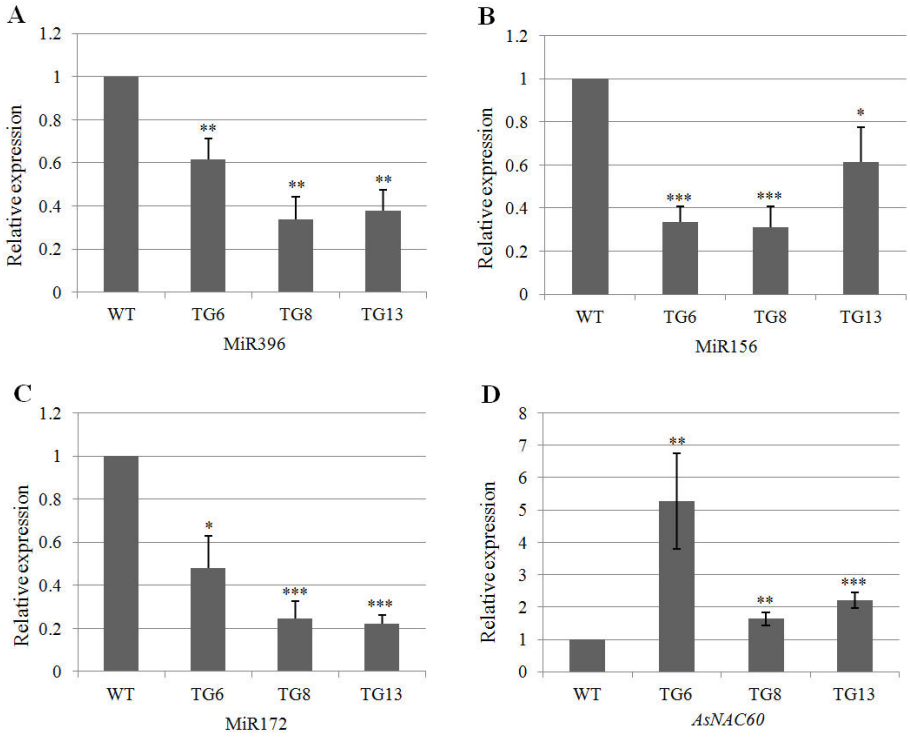


Figure 2.11 Expression levels of the abiotic stress-related miRNAs and *AsNAC60* in WT controls and *Osa-miR528* transgenic lines. Expression levels of A, miR396, B, miR156, and C, miR172 in WT and TG plants revealed through stem-loop RT-qPCR analysis. D, Expression levels of *AsNAC60* in WT and three transgenic lines through RT-qPCR analysis. Three technical replicates were used for the RT-qPCR analysis. *AsUBQ5* was used as an endogenous control. The relative changes of gene expression were

calculated using the $2^{-\Delta\Delta CT}$ method. The error bars represent standard error (n=3). Asterisks (*, ** or ***) indicate significant differences of expression levels between WT control and each transgenic line at $P<0.05$, 0.01 or 0.001 by Student's *t-test*.

Although the importance of miRNAs in the complex stress response network is being gradually recognized, the molecular mechanisms of miRNA-mediated plant stress response are still largely unknown. It would be interesting to know if different miRNAs have interactions in plant response to abiotic stress. To this end, stem-loop RT-qPCR was conducted to analyze several conserved miRNAs which are largely involved in plant abiotic stress response through large scale analysis, including miR396, miR156, and miR172 (Bhardwaj, et al., 2014; Hackenberg, et al., 2012; Pandey, et al., 2014; Wu, et al., 2009a; Xie, et al., 2015; Gao, et al., 2010a; Gupta, et al., 2014; Cui, et al., 2014; Liang, et al., 2012). Figure 2.11A-C shows that miR396, miR156 and miR172 were all down-regulated in transgenic plants overexpressing *Osa-miR528*, suggesting a potential crosstalk of miR528 with other miRNAs in the regulatory network to orchestrate plant response to stress. It should be noted that since these miRNAs are also predominantly involved in development, miR156/miR172 control phase change and flowering time (Huijser and Schmid, 2011), and miR396 controls cell development and proliferation in leaves (Rodriguez, et al., 2010). It is possible that these miRNA changes in the transgenic plants may reflect their crosstalk with miR528 to contribute to the changes in plant development, leading to altered plant morphology observed in miR528 transgenic plants.

Besides its crosstalk with other miRNAs, miR528 was also examined for its potential impact on the expression of other stress-related genes. NAM, ATAF and CUC (NAC)

proteins play an important role in plant environmental stress tolerance (Koyama, et al., 2010). *ONAC60* (*Os12g41680*), one of the NAC family members in rice, is significantly up-regulated by high salinity (Nuruzzaman, et al., 2010). We examined the expression of *AsNAC60*, an ortholog of *ONAC60* in creeping bentgrass (Zhou, et al., 2013). Figure 2.11D shows that *AsNAC60* was up-regulated in three transgenic lines, suggesting that miR528 may indirectly regulate *AsNAC60* expression, which contributes to the concerted plant response to stress.

2.3 Discussion

MiR528-mediated plant development

MiRNAs play diverse roles in plant development. However, there has been no report so far demonstrating the involvement of miR528 in plant development at the vegetative stage growth. In this study, we show that transgenic creeping bentgrass overexpressing *Osa-miR528* exhibits thicker leaves, more vascular bundles, more tillers, shorter internodes and tillers, and more upright growth than WT controls. Less vertical plant growth due to shortened internodes resulting in a dwarf phenotype is one of the desirable traits in turfgrass. Increased tiller number that results in denser, more attractive and uniform communities is another desirable turf trait.

Lignin contributes to the structural rigidity of the cell wall, which is required to keep plants continuously erect. MiR528-expressing transgenic creeping bentgrass has more vascular bundles than WT controls, suggesting that miR528 transgenics might contain more lignin than WT controls. This is supported by the more upright growth of transgenic

plants than WT controls, (Figure A-1C). Further study of the difference in lignin content and cell wall structure between WT and TG plants should provide evidence for a better understanding of miR528-mediated alteration in plant development.

Lodging largely reduces crop yield and grain quality. Strong and short stems are essential in plant lodging resistance. MiR528 transgenic plants with increased vascular bundles and probably higher lignin contents contribute to the stronger stems than WT controls. It is likely that the stronger stems of the TG plants would lead to an enhanced lodging resistance. Additionally, shorter internodes and shorter tiller length of the transgenics would also account for increased lodging resistance. To our knowledge, there have been no studies showing miRNA-mediated plant lodging responses. Our data demonstrate the possible involvement of miR528 in plant response to lodging stress via regulating plant architecture.

ROS scavenging and abiotic stress resistance

Antioxidants such as AA and glutathione, and ROS-scavenging enzymes such as SOD, CAT, and ascorbate peroxidase play important roles in protecting plants against abiotic stresses (Koussevitzky, et al., 2008; Leshem, et al., 2006; Wang, et al., 2005; Rizhsky, et al., 2004). Among the predicted targets of miR528 in rice, AAO, laccase precursor protein, and Zn/Cu SOD are involved in ROS detoxification, and *CBP1* encodes a protein belonging to cupredoxin superfamily, which functions in oxidation homeostasis and electron transfer reactions, suggesting the critical role of miR528 in plant abiotic stress tolerance. In Zn-deficient sorghum (*Sorghum bicolor*), miR528 is

down-regulated in young leaves while the gene expression and the enzyme activity of Zn/Cu SOD are increased (Li, et al., 2013). In transgenic creeping bentgrass overexpressing miR528, the enzyme activity of SOD does not show significant difference between WT and TG plants before and after salinity stress. Further studies by cloning and analyzing genes encoding Zn/Cu SOD will provide information for a better understanding of the interaction between SOD and miR528 in creeping bentgrass.

CAT, mainly localized in peroxisomes, serves as an efficient ROS scavenger to remove excessive H_2O_2 to avoid oxidative damage (Mhamdi, et al., 2010). In this study, TG plants show increased H_2O_2 scavenging, indicating that they have an enhanced capacity of balancing between ROS accumulation and scavenging compared to WT controls, and thus leading to enhanced salinity resistance. Many *CATs* are regulated by developmental and environmental oxidative stresses (Chen, et al., 2012; Du, et al., 2008). In *Capsicum annuum*, a CAT gene is induced under NaCl treatment (Kwon and An, 2001). Among the predicted targets of miR528, however, there is no gene encoding CAT. Presumably, other factors triggered by miR528 overexpression cause the increased CAT activity and contribute to the increased salt tolerance.

Environmental stimuli trigger an oxidative burst mainly caused by the changes in redox state. AA is the major redox buffer in plants, while AAO catalyzes the oxidation of AA to dehydroascorbic acid via dehydroascorbate. Previous studies indicates that the redox state of the AA pool in plants is regulated by AAO (Pignocchi and Foyer, 2003; Pignocchi, et al., 2003). Transgenic tobacco overexpressing AAO leads to increased

ozone sensitivity due to the oxidized AA pool in the apoplast, the first line of defense against ROS (Pignocchi and Foyer, 2003; Sanmartin, et al., 2003). In this study, AAO is predicted to be the direct target of miR528. In addition, TG plants exhibit significantly lower AAO activity than WT controls under salinity and N deficiency conditions (Figure 2.9A), which would presumably result in enhanced capacity of maintaining AA redox state in transgenics, and thereby enhancing plant oxidative stress resistance.

Leaf senescence is an age-dependent and highly regulated degenerative process. Accumulation of ROS is a signal to trigger leaf senescence. In this study, transgenic plants exhibit less degree of leaf senescence than WT controls under normal growth conditions (Figure A-4). Given that miR528 plants show enhanced CAT activity, and consequently, increased capacity of ROS-scavenging, it is therefore plausible to speculate that overexpression of miR528 delays the process of leaf senescence in transgenic plants. Although leaf senescence is a genetically programmed and tightly controlled process, it is also influenced by various environmental cues (Becker and Apel, 1993; Buchanan-Wollaston, et al., 2005). Under salinity and N deficiency, miR528 plants display less degree of leaf senescence than WT controls as observed in normal growth conditions, indicating a decreased leaf senescence rate in TG plants. It is likely that the increased CAT activity and decreased AAO activity in transgenics lead to the enhanced ROS scavenging, and consequently delayed leaf senescence.

K^+: Na^+ ratio and high salinity resistance

Elevated cellular Na^+ contents result in the inhibition of K^+ absorption due to the physiochemical similarity between Na^+ and K^+, and consequently, a lack of discrimination between them during cation transporting and enzyme reactions (Maathuis and Amtmann, 1999). As K^+ participates in many important processes during metabolism, growth, and stress response, it is critical to maintain a high cytosolic K^+: Na^+ ratio. In this study, the K^+ :Na^+ ratio is significantly increased in three transgenic lines in comparison with WT controls under salinity stress due to the increased capacity of maintaining stable K^+ acquisition and distribution (Figure 2.5, F and H), which results in enhanced salt tolerance in miR528 plants. Further gene expression analysis reveals that the stable K^+ acquisition in transgenics is associated with the increased expression of *HAK5*, a high-affinity K^+ transporter (Figure 2.6). Similar results were also reported in rice. *OsHAK5* knockout mutant exhibit severely impaired K^+ influx and transport, decreased K^+: Na^+ ratio, and sensitivity to salt stress compared to WT rice; whereas *OsHAK5* overexpression leads to increased K^+ uptake and K^+: Na^+ ratio, and enhanced salt stress tolerance in transgenic rice plants (Yang, et al., 2014).

Interestingly, miR528 transgenic leaves show significantly higher Na^+ contents than WT controls under normal growth conditions (Figure 2.5A), whereas Na^+ contents are similar in WT and TG plants during salt treatment (Figure 2.5B). The similar phenomena have also been observed previously in other plant species. A study based on more than 300 Arabidopsis accessions shows that plants growing in high saline soil have higher Na^+ accumulation in leaves than other organs (Baxter, et al., 2010). The elevated Na^+ in leaves results from a weak allele of AtHKT1;1, which promotes the accumulation of Na^+

in leaves without Na^+ toxicity (Baxter, et al., 2010). Similarly, a transgenic barley overexpressing *HvHKT2;1* exhibits elevated shoot Na^+ accumulation and enhanced salt stress tolerance (Mian, et al., 2011). We speculate that the elevated Na^+ in miR528 transgenic leaves is the result of a salt inclusion mechanism for osmotic adjustment during salt stress. In this study, the similar accumulation of Na^+ in WT and TG plants might account for the saturated Na^+ environment under severe salt stress treatment. Further test under moderate salt conditions and analysis of the main membrane transporters in transgenic plants overexpressing miR528 would allow a better understanding towards miR528-mediated Na^+ uptake and distribution in plants.

N assimilation and N deficiency tolerance

N is one of the most critical elements for crop productivity. Currently, 67% of the N applied to soil is lost into the environment (Abrol and Raghuram, 2007). Though the form and the amount of N available to plants can be improved via optimizing N management, the efficiency of N utilization has to be tackled biologically. NUE mainly includes N uptake and N assimilation. Many attempts have been made so far towards manipulating key genes in the process of these two steps (Fei, et al., 2003; Ferrario-Mery, et al., 1998; Limami, et al., 1999). However, the success is limited. For example, constitutive expression of NiR in Arabidopsis and tobacco leads to increased transcription of NiR, but decreased enzyme activity of NiR due to post-translational modification (Crété, et al., 1997; Takahashi, et al., 2001). In this study, miR528 transgenic plants exhibit increased transcript levels of *AsNiR* (Figure 2.8A) and enzyme activity (Figure 2.8B), which might

contribute to increased N assimilation efficiency and better NUE. The molecular mechanisms of miR528-mediated *AsNiR* regulation remain elusive. It is hypothesized that the expression of *AsNiR* might be controlled by either the targets of miR528, or some other factors triggered by miR528, or directly by miR528 itself at transcriptional, post-transcriptional or post-translational levels. Interestingly, NiR activity in both WT and TG plants increases under N starvation compared to normal conditions, suggesting the involvement of other factors in the regulation of NiR activity.

Given the improved shoot biomass and total N content in TG plants in comparison with WT controls during N deficiency (Figure 2.7H), it is likely that TG plants have increased N uptake or assimilation, or the total NUE. Expression levels of genes encoding key enzymes of NR, NiR, GS, and GOGAT in the N assimilation pathway have been compared between WT and TG plants. Further study characterizing important nitrate transporters in WT and miR528 transgenic plants will allow a better understanding towards the enhanced tolerance to N deficiency.

Excessive N limits plant uptake of other nutritional elements, such as potassium and phosphorus, and subsequently, results in reduced biomass accumulation compared to N-sufficient conditions as observed in this study (Figure 2.7, B, E and F). Interestingly, miR528 transgenics display significantly higher shoot biomass and total N contents than WT controls under excessive N supply (Figure 2.7, B, D-F and H). It is therefore hypothesized that miR528 might be an important regulator functioning in buffering the imbalanced mineral elements. As discussed above, miR528 promotes *AsHAK5*, which

functions in maintaining the potassium acquisition. It is also likely that other factors impacted by miR528 might be more directly involved in maintaining the homeostasis of different nutritional elements during N-excessive conditions, and subsequently leading to the improved biomass in TG plants.

Molecular mechanisms of miR528-mediated plant salt and N deficiency resistance

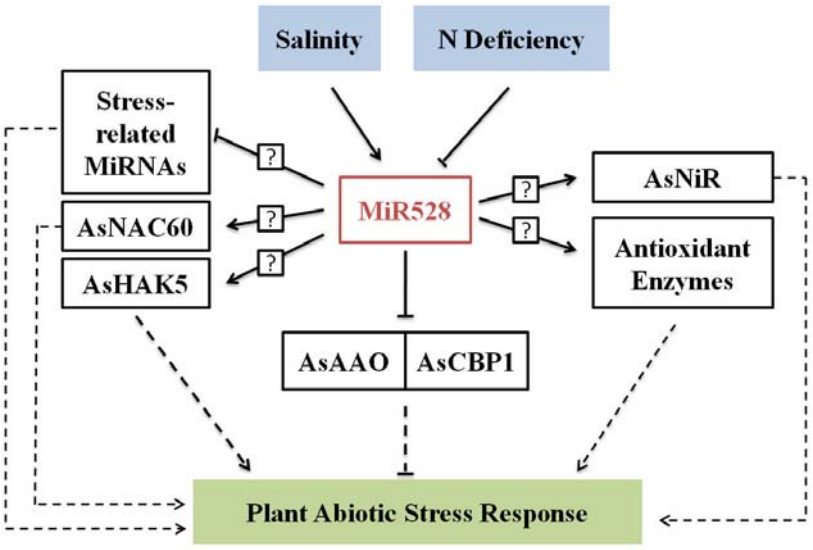


Figure 2.12 A hypothetical model of the molecular mechanisms of miR528-mediated plant abiotic stress response in creeping bentgrass. MiR528 is induced during salinity stress, but down-regulated under N deficiency. MiR528 mediates plant abiotic stress responses through directly repressing the expression of its targets *AsAAO* and *AsCBP1*, which regulate the oxidation homeostasis during abiotic stresses. In addition, miR528 positively regulates *AsNAC60*, *AsHAK5*, *AsNiR* and the gene encoding antioxidant enzyme CAT, which leads to the enhanced tolerance to salinity stress and N deficiency. Furthermore, expression levels of other stress-related miRNAs are negatively regulated by miR528, suggesting that different miRNAs form a regulatory network to coordinately integrate various signals in response to plant abiotic stress.

The accumulation of miR528 is elevated during salt stress (Figure 2.1A), which might cause reduced transcript levels of its predicted targets *AsAAO* and *AsCBP1* (Figure 2.9). Both targets are suggested to mediate oxidation homeostasis and thus prevent damage to cellular components. Besides the direct targets of miR528, genes involved in other

signaling pathways also contribute to the enhanced salt stress tolerance. A high-affinity K^+ transporter AsHAK5, induced in transgenic creeping bentgrass overexpressing miR528 (Figure 2.6), is critical for maintaining the K^+ homeostasis during normal and salinity conditions. Moreover, miR528 induces the activity of CAT, and therefore maintains the ROS homeostasis under abiotic stress. In addition to functional proteins, miR528 also positively regulates *AsNAC60* (Figure 2.11D), which is a creeping bentgrass orthologue of a salt stress-induced transcription factor, suggesting the importance of *AsNAC60* in miR528-mediated salt stress tolerance in creeping bentgrass. MiR528 is gradually repressed during N deficiency (Figure 2.1C), and therefore releasing the inhibition of its targets, which contribute to the oxidation homeostasis. AsNiR, a key enzyme in the N assimilation pathway, is positively regulated by miR528 (Figure 2.8B). The enhanced NUE is presumably attributed to the increased AsNiR activity. MiRNAs are suggested to serve as master regulators in the complex regulatory network of plant response to abiotic stress (Zhou and Luo, 2013). The impact of miR528 on the expression of other stress-related miRNAs observed in this study (Figure 2.11, A-C) suggests coordinated interactions of multiple stress regulators, leading to the enhanced salt and N deficiency tolerance. The hypothetical model of miR528-mediated plant abiotic stress response pathway (Figure 2.12) provides information allowing development of novel molecular strategies to genetically engineer crop species for enhanced environmental stress tolerance.

2.4 Materials and methods

Cloning of the miR528 gene and plasmid construction

The 550 bp rice cDNA (AK073820) containing the *Osa-miR528* stem-loop structure was isolated by PCR using the forward and reverse primer set 5'-TCTAGAGATCAGCAGCAGCCACA-3' and 5'-GTCGACGACCAAATAATGTGTT ACTG-3', which contained an XbaI and a SalI restriction sites (underlined), respectively. PCR products were cloned into the binary vector pZH01 (Xiao, et al., 2003), generating the *Osa-miR528* overexpression gene construct, p35S-*Osa-miR528*/p35S-*hyg*. The construct contains the cauliflower mosaic virus 35S (CaMV 35S) promoter driving *Osa-miR528* linked to the CaMV 35S promoter driving the *hyg* gene for hygromycin resistance as a selectable marker. For subsequent plant transformation, the construct was transferred into *Agrobacterium tumefaciens* strain LBA4404.

Plant materials and transformation

Creeping bentgrass cultivar 'Penn A-4' (supplied by HybriGene) was used for plant transformation. Transgenic plants constitutively expressing *Osa-miR528* were produced via *Agrobacterium*-mediated transformation of embryonic callus induced from mature seeds as described previously (Luo, et al., 2004).

Plant propagation, maintenance, and abiotic stress treatments

The regenerated transgenic plants overexpressing *Osa-miR528* and WT controls were clonally propagated from tillers and maintained as described previously (Zhou, et al., 2013).

For salt stress treatments, plants grown in cone-tainers were immersed in the 200 mM NaCl solution supplemented with 0.2 g/L water-soluble fertilizer. After a nine-day salt treatment, shoots and roots were harvested for further physiological analysis. Replicates of the salt-treated plants have recovered from salt stress by watering with 0.2 g/L water-soluble fertilizer every other day and were photographed for documentation. For miR528 expression analysis, WT leaves were collected at 0, 1.5, 3, and 6 h after salt stress treatment.

To test the performance of WT and TG plants under different concentrations of N, plants grown in cone-tainers were immersed in modified MS nutrient solution of pH 5.7 containing 3 mM $CaCl_2$•$2H_2O$, 1.5 mM $MgSO_4$•$7H_2O$, 1.25 mM KH_2PO_4, 0.1 mM H_3BO_3, 0.1 mM $MnSO_4$•$4H_2O$, 0.1 mM $ZnSO_4$•H_2O, 0.5 μM KI, 0.56 μM $NaMO_4$•2 H_2O, 0.1 μM $CuSO_4$•$5H_2O$, 0.1 μM $CoCl_2$•$6H_2O$, 0.1 mM $FeSO_4$•$7H_2O$, 0.1 mM Na_2EDTA•$2H_2O$, supplemented with N of different concentrations (0.4, 2, 10 or 40 mM). N supplies include KNO_3 and NH_4NO_3. Equivalent amounts of KCl were added to the nutrient solutions to maintain the osmotic potential and prevent potassium deficiency caused by low supplies of KNO_3. For miR528 expression analysis, WT plants were immersed in modified MS nutrient solution without N. WT leaves were collected at 0, 2, 5, 8 and 12 d after N starvation treatment.

The expression profile of miR528 during drought stress was analyzed in WT creeping bentgrass leaves. WT plants grew hydroponically with 0.2 g/L water-soluble fertilizer. Right before the drought treatment, the plants were taken out of the nutritional solution

and dried with paper towel. They were then laid down on the bench for air-drying. The leaf samples were collected at 0, 1, 2 and 4 h after drought treatment.

Plant DNA, RNA isolation and expression analysis

Plant genomic DNA was extracted from 30 mg of fresh leaves following Luo's protocol (Luo, et al., 1995). Plant total RNA was isolated from 100 mg of fresh leaves using Trizol reagent (Invitrogen) following the manufacturer's protocol. First strand cDNA was synthesized from 2 μg of RNA with SuperScript II Reverse Transcriptase (Invitrogen) and oligo (dT) or gene specific primers. The semi-quantitative RT-PCR was conducted on 24 to 30 cycles based on its exponential phase. PCR products were separated by electrophoresis using 0.8% or 1.5% agarose gel, visualized and photographed with BioDoc-It imaging system (UVP LLC).

Quantitative RT-PCR (RT-qPCR) was performed with 12.5μL of iQ SYBR-Green Super-mix (Bio-Rad Laboratories) per 25 μL reaction system. The green fluorescence signal was monitored on the Bio-Rad iQ5 real-time detection system by using iQ5 Optical System Software version 2.0 (Bio-Rad Laboratories). *AsACT1* (JX644005) and *AsUBQ5* (JX570760) were used as endogenous controls. The relative changes of gene expression were calculated based on the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Stem-loop RT-qPCR was performed according to the protocol by Varkonyi-Gasicet al. (Varkonyi-Gasic, et al., 2007). The *Osa-miR528* stem-loop RT primer and PCR forward primer are 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGA TACGACCTCCTC-3' and 5'-GCAGTGGAAGGGGCATGCA-3', respectively.

Measurement of mineral content

For Na^+ and K^+ content measurements, leaves and roots of WT and transgenic plants were collected before and after a nine-day 200 mM NaCl solution treatment. For total N measurement, WT and transgenic plant leaves were collected after four weeks of treatments with different concentrations of N, including 0.4, 2, 10 and 40 mM. 0.2 gram of each dried sample for total N measurement and 0.5 gram of each dried sample for Na^+ and K^+ content measurements were analyzed to determine the mineral content according to previous protocols (Haynes, 1980; Li, et al., 2010).

Measurement of leaf RWC, EL, chlorophyll and proline contents

Plant leaf RWC, EL, chlorophyll a and b, as well as proline contents were measured following previous protocols (Bates, et al., 1973; Li, et al., 2010).

Antioxidant enzyme assay

A total of 0.1 gram of leaves were weighed and homogenized immediately in 1 mL of 0.05 M Phosphate Buffered Saline at pH 7.8 on ice and centrifuged at 14,000 × g for 30 min at 4 °C. The supernatant of each sample was transferred to a new 1.5 mL eppendorf tube and stored on ice for further enzyme assay. The activity of SOD (EC 1.15.1.1) was determined as described previously (Giannopolitis and Ries, 1977). One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% reduction rate of nitro blue tetrazolium chloride.

The activity of CAT (EC 1.11.7.6) was determined as described previously (Maehly and Chance, 1954). The decomposition of H_2O_2 was measured by the decrease of absorbance at 240 nm for 1 min. An enzyme unit was defined as the amount of enzyme necessary to decompose 1 μM of H_2O_2 at 25°C in 1 min.

NiR assay (EC 1.7.2.1)

NiR activity was measured by a spectrophotometric assay as described previously (Losada and Paneque, 1971). The nitrite content was calculated from a KNO_2 standard curve. NiR activity was determined by the reduction of nitrite that was catalyzed by the enzyme in 1 mg of soluble protein per hour. Protein contents were measured according to the Bradford Dye-binding method (Bradford, 1976).

AAO assay (EC 1.10.3.3)

The activity of AAO was determined spectrophotometrically at 25°C following the decrease in absorbance of ascorbic acid (AsA) at 265 nm as described previously (Esaka, et al., 1988). The activity of AAO was determined as:

Activity ($U \cdot mg^{-1}$protein) = (ΔA_{265} /min) × 2 × dilution factor/(e × 0.05 × total protein) [e = 13.386 $mM^{-1}cm^{-1}$ (an extinction coefficient for AsA at 265 nm); total volume = 2 mL; enzyme volume = 0.05 mL]

Plant histology analysis

The second and third internodes from the top tillers and the fully expanded leaves of WT and TG plants were collected and immersed in formalin-acetic-alcohol fixation for

48 hours at room temperature. After fixation, plant tissues were dehydrated with a series of graded ethanol from 70% to 100%, followed by paraffin wax infiltration. Tissues were then embedded in paraffin blocks to process section using the rotary microtome (RM 2165, Leica). Sections were stained using toluidine blue and observed under stereo microscope (MEIJI EM-5). Photographs were taken using 35 mm SLR camera body (Canon) connected to a microscope. Scale bars were added to photographs using ImageJ (Abràmoff, et al., 2004).

Sequence data from this article can be found in GenBank under accession numbers KR911823, KR911824, KR911825, KR911826, KR911827, KR911828, and KR911829.

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References

Abràmoff MD, Magalhães PJ, Ram SJ (2004) Image processing with ImageJ. Biophoton Int **11:** 36-43

Abrol Y, Raghuram N (2007) Agricultural nitrogen use and its environmental implications. IK International Publishing House, New Dehli, India

Ali Y, Aslam Z, Ashraf M, Tahir G (2004) Effect of salinity on chlorophyll concentration, leaf area, yield and yield components of rice genotypes grown under saline environment. Int J Environ Sci Technol **1:** 221-225

An F, Hsiao S, Chan M (2011) Sequencing-based approaches reveal low ambient temperature-responsive and tissue-specific microRNAs in *Phalaenopsis orchid*. PLoS One **6:** e18937

Ashraf M, Foolad M (2007) Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ Exp Bot **59:** 206-216

Bates L, Waldren R, Teare I (1973) Rapid determination of free proline for water-stress studies. Plant Soil **39:** 205-207

Baxter I, Brazelton JN, Yu D, Huang YS, Lahner B, Yakubova E, Li Y, Bergelson J, Borevitz JO, Nordborg M (2010) A coastal cline in sodium accumulation in *Arabidopsis thaliana* is driven by natural variation of the sodium transporter AtHKT1; 1. PLoS Genet **6:** e1001193

Becker W, Apel K (1993) Differences in gene expression between natural and artificially induced leaf senescence. Planta **189:** 74-79

Bhardwaj AR, Joshi G, Pandey R, Kukreja B, Goel S, Jagannath A, Kumar A, Katiyar-Agarwal S, Agarwal M (2014) A Genome-Wide Perspective of miRNAome in Response to High Temperature, Salinity and Drought Stresses in *Brassica juncea* (Czern) L. PloS ONE **9:** e92456

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248-254

Buchanan-Wollaston V, Page T, Harrison E, Breeze E, Lim PO, Nam HG, Lin J, Wu S, Swidzinski J, Ishizaki K (2005) Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in Arabidopsis. Plant J **42:** 567-585

Chapin III FS, Walter CH, Clarkson DT (1988) Growth response of barley and tomato to nitrogen stress and its control by abscisic acid, water relations and photosynthesis. Planta **173:** 352-366

Chen H, Wu S, Huang G, Shen C, Afiyanti M, Li W, Lin Y (2012) Expression of a cloned sweet potato catalase SPCAT1 alleviates ethephon-mediated leaf senescence and H_2O_2 elevation. J Plant Physiol **169:** 86-97

Crété P, Caboche M, Meyer C (1997) Nitrite reductase expression is regulated at the post-transcriptional level by the nitrogen source in Nicotiana plumbaginifolia and *Arabidopsis thaliana*. Plant J **11:** 625-634

Cui L, Shan J, Shi M, Gao J, Lin H (2014) The miR156-SPL9-DFR pathway coordinates the relationship between development and abiotic stress tolerance in plants. Plant J **80:** 1108-1117

Dai X, Zhao PX (2011) psRNATarget: a plant small RNA target analysis server. Nucleic Acids Res **39:** W155-9

Dennison C (2005) Investigating the structure and function of cupredoxins. Coord Chem Rev **249:** 3025-3054

Ding D, Zhang L, Wang H, Liu Z, Zhang Z, Zheng Y (2009) Differential expression of miRNAs in response to salt stress in maize roots. Ann Bot **103:** 29-38

Du Y, Wang P, Chen J, Song C (2008) Comprehensive functional analysis of the catalase gene family in *Arabidopsis thaliana*. J Integr Plant Biol **50:** 1318-1326

Esaka M, Imagi J, Suzuki K, Kubota K (1988) Formation of ascorbate oxidase in cultured pumpkin cells. Plant Cell Physiol **29:** 231-235

Fei H, Chaillou S, Hirel B, Mahon JD, Vessey KJ (2003) Overexpression of a soybean cytosolic glutamine synthetase gene linked to organ-specific promoters in pea plants grown in different concentrations of nitrate. Planta **216:** 467-474

Ferrario-Mery S, Valadier MH, Foyer CH (1998) Overexpression of nitrate reductase in tobacco delays drought-induced decreases in nitrate reductase activity and mRNA. Plant Physiol **117:** 293-302

Ferreira TH, Gentile A, Vilela RD, Costa GGL, Dias LI, Endres L, Menossi M (2012) microRNAs associated with drought response in the bioenergy crop sugarcane (*Saccharum* spp.). PLoS ONE **7:** e46703

Fraisier V, Gojon A, Tillard P, Daniel-Vedele F (2000) Constitutive expression of a putative high-affinity nitrate transporter in *Nicotiana plumbaginifolia*: evidence for post-transcriptional regulation by a reduced nitrogen source. Plant J **23:** 489-496

Gambale F, Uozumi N (2006) Properties of Shaker-type potassium channels in higher plants. J Membr Biol **210:** 1-19

Gao P, Bai X, Yang L, Lv D, Li Y, Cai H, Ji W, Guo D, Zhu Y (2010) Over-expression of osa-MIR396c decreases salt and alkali stress tolerance. Planta **231:** 991-1001

Giannopolitis CN, Ries SK (1977) Superoxide dismutases: I. Occurrence in higher plants. Plant Physiol **59:** 309-314

Golldack D, Lüking I, Yang O (2011) Plant tolerance to drought and salinity: stress regulating transcription factors and their functional significance in the cellular transcriptional network. Plant Cell Rep **30:** 1383-1391

Gupta OP, Meena NL, Sharma I, Sharma P (2014) Differential regulation of microRNAs in response to osmotic, salt and cold stresses in wheat. Mol Biol Rep **41:** 4623-4629

Hackenberg M, Shi B, Gustafson P, Langridge P (2012) A transgenic transcription factor (TaDREB3) in barley affects the expression of microRNAs and other small non-coding RNAs. PloS ONE **7:** e42030

Haynes R (1980) A comparison of two modified Kjeldahl digestion techniques for multi-element plant analysis with conventional wet and dry ashing methods. Commun Soil Sci Plant Anal **11:** 459-467

Huijser P, Schmid M (2011) The control of developmental phase transitions in plants. Development **138:** 4117-4129

Kandlbinder A, Finkemeier I, Wormuth D, Hanitzsch M, Dietz K (2004) The antioxidant status of photosynthesizing leaves under nutrient deficiency: redox regulation, gene expression and antioxidant activity in *Arabidopsis thaliana*. Physiol Plant **120:** 63-73

Kikuchi S, Satoh K, Nagata T, Kawagashira N, Doi K, Kishimoto N, Yazaki J, Ishikawa M, Yamada H, Ooka H Foundation of Advancement of International Science Genome Sequencing & Analysis Group, RIKEN (2003) Collection, mapping, and annotation of over 28,000 cDNA clones from japonica rice. Science **301:** 376-379

Kim M, Sato S, Sasaki K, Saburi W, Matsui H, Imai R (2013) COLD SHOCK DOMAIN PROTEIN 3 is involved in salt and drought stress tolerance in Arabidopsis. FEBS Open Bio **3:** 438-442

Koussevitzky S, Suzuki N, Huntington S, Armijo L, Sha W, Cortes D, Shulaev V, Mittler R (2008) Ascorbate peroxidase 1 plays a key role in the response of *Arabidopsis thaliana* to stress combination. J Biol Chem **283:** 34197-34203

Koyama T, Mitsuda N, Seki M, Shinozaki K, Ohme-Takagi M (2010) TCP transcription factors regulate the activities of ASYMMETRIC LEAVES1 and miR164, as well as the auxin response, during differentiation of leaves in Arabidopsis. Plant Cell **22:** 3574-3588

Kozomara A, Griffiths-Jones S (2014) miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res **42:** D68-73

Kwon SI, An CS (2001) Molecular cloning, characterization and expression analysis of a catalase cDNA from hot pepper (Capsicum annuum L.). Plant Sci **160:** 961-969

Lebaudy A, Véry A, Sentenac H (2007) K channel activity in plants: genes, regulations and functions. FEBS Lett **581:** 2357-2366

Leshem Y, Melamed-Book N, Cagnac O, Ronen G, Nishri Y, Solomon M, Cohen G, Levine A (2006) Suppression of Arabidopsis vesicle-SNARE expression inhibited fusion of H_2O_2-containing vesicles with tonoplast and increased salt tolerance. Proc Natl Acad Sci USA **103:** 18008-18013

Li C, Chang P, Ghebremariam KM, Qin L, Liang Y (2014) Overexpression of tomato SpMPK3 gene in Arabidopsis enhances the osmotic tolerance. Biochem Biophys Res Commun **443:** 357-362

Li Y, Zhang Y, Shi D, Liu X, Qin J, Ge Q, Xu L, Pan X, Li W, Zhu Y (2013) Spatial-temporal analysis of zinc homeostasis reveals the response mechanisms to acute zinc deficiency in *Sorghum bicolor*. New Phytol **200:** 1102-1115

Li Z, Baldwin CM, Hu Q, Liu H, Luo H (2010) Heterologous expression of Arabidopsis H -pyrophosphatase enhances salt tolerance in transgenic creeping bentgrass (*Agrostis stolonifera* L.). Plant, Cell Environ **33:** 272-289

Liang G, He H, Yu D (2012) Identification of nitrogen starvation-responsive microRNAs in *Arabidopsis thaliana*. PloS One **7**: e48951

Limami A, Phillipson B, Ameziane R, Pernollet N, Jiang Q, Roy R, Deleens E, Chaumont-Bonnet M, Gresshoff PM, Hirel B (1999) Does root glutamine synthetase control plant biomass production in *Lotus japonicus* L.? Planta **209:** 495-502

Liu B, Li P, Li X, Liu C, Cao S, Chu C, Cao X (2005) Loss of function of OsDCL1 affects microRNA accumulation and causes developmental defects in rice. Plant Physiol **139:** 296-305

Livak K, Schmittgen T (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods **25:** 402-408

Losada M, Paneque A (1971) Nitrite reductase. Meth Enzymol **23:** 487-491

Lu Y, Que L, Tolman W (2004) Biocoordination Chemistry. Comprehensive Coordination Chemistry II: From Biology to Nanotechnology Vol 8. Elsevier, Oxford, pp 91-122

Lu Y, Li Y, Zhang J, Xiao Y, Yue Y, Duan L, Zhang M, Li Z (2013) Overexpression of Arabidopsis molybdenum cofactor sulfurase gene confers drought tolerance in maize (Zea mays L.). PloS ONE **8:** e52126

Luo H, Hu Q, Nelson K, Longo C, Kausch AP, Chandlee JM, Wipff JK, Fricker CR (2004) *Agrobacterium tumefaciens*-mediated creeping bentgrass (*Agrostis stolonifera* L.) transformation using phosphinothricin selection results in a high frequency of single-copy transgene integration. Plant Cell Rep **22:** 645-652

Luo H, Van Coppenolle B, Seguin M, Boutry M (1995) Mitochondrial DNA polymorphism and phylogenetic relationships in *Hevea brasiliensis*. Mol Breed **1:** 51-63

Maathuis FJ, Amtmann A (1999) K nutrition and Na toxicity: the basis of cellular K /Na ratios. Annals of Botany **84:** 123-133

Maehly A, Chance B (1954) Catalases and peroxidases. Methods Biochem Anal **1:** 357-424

Marschner H (1995) Functions of mineral nutrients: macronutrients. Mineral nutrition of higher plants **2:** 379-396

Marshall NM, Garner DK, Wilson TD, Gao Y, Robinson H, Nilges MJ, Lu Y (2009) Rationally tuning the reduction potential of a single cupredoxin beyond the natural range. Nature **462:** 113-116

Masclaux-Daubresse C, Daniel-Vedele F, Dechorgnat J, Chardon F, Gaufichon L, Suzuki A (2010) Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. Ann Bot **105:** 1141-1157

Maser P, Thomine S, Schroeder JI, Ward JM, Hirschi K, Sze H, Talke IN, Amtmann A, Maathuis FJ, Sanders D, Harper JF, Tchieu J, Gribskov M, Persans MW, Salt DE, Kim SA, Guerinot ML (2001) Phylogenetic relationships within cation transporter families of Arabidopsis. Plant Physiol **126:** 1646-1667

Mhamdi A, Queval G, Chaouch S, Vanderauwera S, Van Breusegem F, Noctor G (2010) Catalase function in plants: a focus on Arabidopsis mutants as stress-mimic models. J Exp Bot **61:** 4197-4220

Mian A, Oomen RJ, Isayenkov S, Sentenac H, Maathuis FJ, Véry A (2011) Over-expression of an Na^+ -and K^+-permeable HKT transporter in barley improves salt tolerance. Plant J **68:** 468-479

Munns R (2002) Comparative physiology of salt and water stress. Plant, Cell Environ **25:** 239-250

Munns R (1993) Physiological processes limiting plant growth in saline soils: some dogmas and hypotheses. Plant, Cell Environ **16:** 15-24

Nischal L, Mohsin M, Khan I, Kardam H, Wadhwa A, Abrol YP, Iqbal M, Ahmad A (2012) Identification and comparative analysis of microRNAs associated with low-N tolerance in rice genotypes. PloS ONE **7:** e50261

Nuruzzaman M, Manimekalai R, Sharoni AM, Satoh K, Kondoh H, Ooka H, Kikuchi S (2010) Genome-wide analysis of NAC transcription factor family in rice. Gene **465:** 30-44

Pandey R, Joshi G, Bhardwaj AR, Agarwal M, Katiyar-Agarwal S (2014) A comprehensive genome-wide study on tissue-specific and abiotic stress-specific miRNAs in *Triticum aestivum*. PloS ONE **9:** e95800

Pathak RR, Ahmad A, Lochab S, Raghuram N (2008) Molecular physiology of plant nitrogen use efficiency and biotechnological options for its enhancement. Current Science Bangalore **94:** 1394

Pignocchi C, Foyer CH (2003) Apoplastic ascorbate metabolism and its role in the regulation of cell signalling. Curr Opin Plant Biol **6:** 379-389

Pignocchi C, Fletcher JM, Wilkinson JE, Barnes JD, Foyer CH (2003) The function of ascorbate oxidase in tobacco. Plant Physiol **132:** 1631-1641

Rao G, Rao GR (1981) Pigment composition and chlorophyllase activity in pigeon pea (*Cajanus indicus Spreng*) and Gingelley (*Sesamum indicum* L.) under NaCl salinity. Indian J Exp Biol **19**:768-770

Rizhsky L, Liang H, Shuman J, Shulaev V, Davletova S, Mittler R (2004) When defense pathways collide. The response of Arabidopsis to a combination of drought and heat stress. Plant Physiol **134:** 1683-1696

Rodriguez RE, Mecchia MA, Debernardi JM, Schommer C, Weigel D, Palatnik JF (2010) Control of cell proliferation in *Arabidopsis thaliana* by microRNA miR396. Development **137:** 103-112

Sanmartin M, Drogoudi PD, Lyons T, Pateraki I, Barnes J, Kanellis AK (2003) Over-expression of ascorbate oxidase in the apoplast of transgenic tobacco results in altered ascorbate and glutathione redox states and increased sensitivity to ozone. Planta **216:** 918-928

Satoh K, Doi K, Nagata T, Kishimoto N, Suzuki K, Otomo Y, Kawai J, Nakamura M, Hirozane-Kishikawa T, Kanagawa S, Arakawa T, Takahashi-Iida J, Murata M, Ninomiya N, Sasaki D, Fukuda S, Tagami M, Yamagata H, Kurita K, Kamiya K, Yamamoto M, Kikuta A, Bito T, Fujitsuka N, Ito K, Kanamori H, Choi IR, Nagamura Y, Matsumoto T, Murakami K, Matsubara K, Carninci P, Hayashizaki Y, Kikuchi S (2007) Gene organization in rice revealed by full-length cDNA mapping and gene expression analysis through microarray. PLoS ONE **2:** e1235

Sharma D, Tiwari M, Lakhwani D, Tripathi RD, Trivedi PK (2015) Differential expression of microRNAs by arsenate and arsenite stress in natural accessions of rice. Metallomics 7: 174-187

Shinozaki K, Yamaguchi-Shinozaki K (2007) Gene networks involved in drought stress response and tolerance. J Exp Bot **58:** 221-227

Solomon EI, Szilagyi RK, DeBeer George S, Basumallick L (2004) Electronic structures of metal sites in proteins and models: contributions to function in blue copper proteins. Chem Rev **104:** 419-458

Takahashi M, Sasaki Y, Ida S, Morikawa H (2001) Nitrite reductase gene enrichment improves assimilation of NO_2 in Arabidopsis. Plant Physiol **126:** 731-741

Thiebaut F, Rojas CA, Grativol C, Motta MR, Vieira T, Regulski M, Martienssen RA, Farinelli L, Hemerly AS, Ferreira PC (2014) Genome-wide identification of microRNA and siRNA responsive to endophytic beneficial diazotrophic bacteria in maize. BMC Genomics **15:** 766-2164-15-766

Turan S, Cornish K, Kumar S (2012) Salinity tolerance in plants: breeding and genetic engineering. Aust J Crop Sci **6**: 1337–1348

Varkonyi-Gasic E, Wu R, Wood M, Walton EF, Hellens RP (2007) Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs. Plant methods **3:** 1-12

Véry A, Sentenac H (2003) Molecular mechanisms and regulation of K transport in higher plants. Annu Rev Plant Biol **54:** 575-603

Véry A, Sentenac H (2002) Cation channels in the Arabidopsis plasma membrane. Trends Plant Sci **7:** 168-175

Wang F, Wang Q, Kwon S, Kwak S, Su W (2005) Enhanced drought tolerance of transgenic rice plants expressing a pea manganese superoxide dismutase. J Plant Physiol **162:** 465-472

Wu G, Park MY, Conway SR, Wang J, Weigel D, Poethig RS (2009a) The sequential action of miR156 and miR172 regulates developmental timing in Arabidopsis. Cell **138:** 750-759

Wu L, Zhang Q, Zhou H, Ni F, Wu X, Qi Y (2009b) Rice MicroRNA effector complexes and targets. Plant Cell **21:** 3421-3435

Xiao H, Wang Y, Liu D, Wang W, Li X, Zhao X, Xu J, Zhai W, Zhu L (2003) Functional analysis of the rice AP3 homologue OsMADS16 by RNA interference. Plant Mol Biol **52:** 957-966

Xie F, Wang Q, Sun R, Zhang B (2015) Deep sequencing reveals important roles of microRNAs in response to drought and salinity stress in cotton. J Exp Bot **66:** 789-804

Xu Z, Zhong S, Li X, Li W, Rothstein SJ, Zhang S, Bi Y, Xie C (2011) Genome-wide identification of microRNAs in response to low nitrate availability in maize leaves and roots. PloS ONE **6:** e28009

Yan J, Zhang H, Zheng Y, Ding Y (2015) Comparative expression profiling of miRNAs between the cytoplasmic male sterile line MeixiangA and its maintainer line MeixiangB during rice anther development. Planta **241:** 109-123

Yang T, Zhang S, Hu Y, Wu F, Hu Q, Chen G, Cai J, Wu T, Moran N, Yu L, Xu G (2014) The role of a potassium transporter OsHAK5 in potassium acquisition and transport from roots to shoots in rice at low potassium supply levels. Plant Physiol **166:** 945-959

Yeo A (1998) Molecular biology of salt tolerance in the context of whole-plant physiology. J Exp Bot **49:** 915-929

Zanca AS, Vicentini R, Ortiz-Morea FA, Del Bem LE, da Silva MJ, Vincentz M, Nogueira FT (2010) Identification and expression analysis of microRNAs and targets in the biofuel crop sugarcane. BMC Plant Biol **10:** 260-2229-10-260

Zhang Z, Wei L, Zou X, Tao Y, Liu Z, Zheng Y (2008) Submergence-responsive MicroRNAs are potentially involved in the regulation of morphological and metabolic adaptations in maize root cells. Ann Bot **102:** 509-519

Zhou M, Luo H (2013) MicroRNA-mediated gene regulation: potential applications for plant genetic engineering. Plant Mol Biol **83:** 59-75

Zhou M, Li D, Li Z, Hu Q, Yang C, Zhu L, Luo H (2013) Constitutive expression of a miR319 gene alters plant development and enhances salt and drought tolerance in transgenic creeping bentgrass. Plant Physiol **161:** 1375-1391

Zhu JK (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol **53:** 247-273

CHAPTER III - CONSTITUTIVE EXPRESSION OF MIR396C ALTERS PLANT DEVELOPMENT AND ENHANCES SALT STRESS TOLERANCE IN TRANSGENIC CREEPING BENTGRASS

Abstract

The conserved microRNA miR396 is involved in plant growth, development, and abiotic stress response in multiple plant species through regulating its targets, *Growth Regulating Factor* transcription factor genes. However, the role of miR396 has not yet been characterized in perennial monocot species. In addition, the molecular mechanism of miR396-mediated abiotic stress response remains unclear. To elucidate the role of miR396 in perennial monocot species, we generated transgenic creeping bentgrass (*Agrostis stolonifera*) overexpressing *Osa-miR396c*, a rice miRNA. Transgenic plants exhibited altered development, including less shoot and root biomass, shorter internodes, smaller leaf area, fewer leaf veins and epidermis cells per unit area than those of wild-type controls. In addition, transgenics showed enhanced salt tolerance associated with improved water and chlorophyll retention, cell membrane integrity, and Na^+ exclusion during high salinity exposure. Four potential targets of miR396 were identified in creeping bentgrass and up-regulated in response to salt stress. RNA-seq analysis indicates that miR396-mediated salt stress tolerance requires the coordination of stress-related functional proteins (antioxidant enzymes and Na^+/H^+ antiporter) and regulatory proteins (transcription factors and protein kinases). This study establishes a molecular pathway to

connect the upstream regulatory and downstream functional elements, and provides insight into the miRNA-mediated regulatory networks.

3.1 Introduction

Soil salinity is a major constraint for crop productivity. High Na^+ content during salt stress results in ionic stress because of the disturbed intracellular ion homeostasis (Hasegawa, et al., 2000). Excess Na^+ negatively affects K^+ uptake because of their similar chemical properties, and thereby leads to the inhibition of many K^+-dependent biological processes, such as protein synthesis, enzymatic reactions, and photosynthesis. To alleviate ionic stress and maintain a high K^+: Na^+ ratio, plants have evolved adaptive strategies of cytosolic Na^+ exclusion and vacuolar Na^+ sequestration. Currently, several classes of Na^+ transporters have been identified to play central roles in these adaptive strategies during high salinity exposure (Uozumi and Schroeder, 2010; Apse and Blumwald, 2007; Hauser and Horie, 2010). The best-characterized Na^+ transporters which alleviate excess Na^+ through compartmentalizing Na^+ into the vacuoles are NHX transporters via mediating intracellular Na^+/H^+ and K^+/H^+ antiport (Blumwald, 2000). Studies in various plant species show that overexpression of NHX genes confers enhanced high salinity tolerance and elevated relative Na^+ content in plant tissue (Apse, et al., 1999; Agarwal, et al., 2013; Zhang, et al., 2001). Besides Na^+ sequestration, the mechanism of Na^+ exclusion has been well elucidated through characterization of salinity overly sensitive 1 (SOS1) transporters, which mediate Na^+/H^+ antiport across the plasma membrane (Wu, et al., 1996). The SOS signaling pathway in response to salt stress has been proposed in Arabidopsis, in which the calcium binding protein AtSOS3 activates the

kinase activity of AtSOS2 to further activate AtSOS1 through the direct phosphorylation (Qiu, et al., 2002; Quan, et al., 2007; Quintero, et al., 2011). Overexpression of *AtSOS1* has been shown to lead to enhanced salt tolerance in transgenic Arabidopsis (Yang, et al., 2009b).

Salt stress also induces osmotic stress due to the accumulation of Na^+ in the apoplast, thereby elevating osmotic gradient between inside and outside of the cells. In response to osmotic stress, certain organic solutes called osmoprotenctants (such as proline, sugars, mannitol etc.) are produced in the cytoplasm to maintain cell turgor pressure (Ben Ahmed, et al., 2010). A previous study indicates that transgenic rice accumulating sugar trehalose exhibits enhanced abiotic stress tolerance (Garg, et al., 2002).

In addition, salt stress leads to enhanced production of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, hydroxyl radical and singlet oxygen, which trigger cell oxidative damage and ultimately cell death (Sharma, et al., 2012). Antioxidant enzymes, such as superoxide dismutase, catalase, guaiacol peroxidase, ascorbate peroxidase, dehydroascorbate reductase, and glutathione reductase, play an essential role in scavenging the overproduced ROS. Overexpression of genes encoding these antioxidants confers enhanced tolerance to salt and other abiotic stresses in a variety of plant species (Gupta, et al., 1993; Guan, et al., 2009; Eltayeb, et al., 2007).

Besides these functional proteins, regulatory roles of transcription factors (TFs), protein kinases, and phosphatases have also been well documented in the plant response to salt stress. Recently, the regulatory role of microRNAs (miRNAs) in plant response to

salt and other environmental stresses has been gradually revealed. For example, transgenic Arabidopsis with higher expression levels of miR408 exhibits enhanced tolerance to salt, cold, and oxidative stresses, but reduced tolerance to drought and osmotic stresses (Ma, et al., 2015). MiR408-mediated plant abiotic stress response is associated with enhanced cellular antioxidant capacity and reduced ROS production (Ma, et al., 2015). Another recent study shows that constitutive expression of *Osa-miR528* confers enhanced salt and nitrogen-deficiency tolerance in transgenic creeping bentgrass, which is associated with up-regulation of K^+ transporter gene *HAK5*, the increased activities of nitrite reductase and antioxidant enzymes, altered expression of other stress-related TF and small RNAs, and the repression of its direct targets (Yuan, et al., 2015). These results strongly suggest that miRNAs coordinate multiple stress-responsive pathways to cope with abiotic stress.

MiR396 is a conserved miRNA, and presents in both monocots and dicots. It performs post-transcriptional gene regulation through repressing its targets, *Growth Regulating Factors* (*GRFs*). The role of miR396 in plant growth and development has been well characterized. Morphologically, transgenic plants constitutively expressing miR396 display shorter plants and narrower leaves than WT controls in Arabidopsis and tobacco because of reduced cell numbers in leaf (Kim, et al., 2003; Yang, et al., 2009a; Rodriguez, et al., 2010). A similar phenotype was observed in *atgrf1 atgrf2 atgrf3* triple mutants (Kim, et al., 2003). In addition, transgenic tobacco overexpressing miR396 showed cotyledon fusion and lack of a shoot apical meristem (Baucher, et al., 2013). Recent study shows that *AtGRF5* participates in the control of leaf senescence

(Debernardi, et al., 2014). Transgenic Arabidopsis overexpressing miR396 causes an early senescence phenotype (Debernardi, et al., 2014). MiR396 is also involved in plant response to various abiotic stresses. *At-miR396* was induced under UV-B radiation resulting in inhibited cell division in proliferating tissues (Casadevall, et al., 2013). Transcription of tomato *miR396a* (*Sp-miR396*) is up-regulated under salt and drought stresses (Young, et al., 2010; Chen, et al., 2015). Transgenic tobacco overexpressing *Sp-miR396a* exhibits enhanced stress resistance to salt, drought, and cold due to the improved osmoregulation and decreased accumulation of reactive oxygen species (ROS) (Chen, et al., 2015). In contrast, transgenic rice and Arabidopsis constitutively expressing *Osa-miR396c* results in reduced salt and alkali stress tolerance in comparison with wild-type plants (Gao, et al., 2010). The opposite responses to salt stress in different plant species suggest a species-specific function of miR396. Currently, the role of miR396 has not been elucidated in perennial monocot species. In addition, the underlying molecular mechanisms of miR396-mediated plant resistance to salt and other environmental stresses remain unclear.

In this study, we generated transgenic (TG) creeping bentgrass plants constitutively expressing rice miR396 to investigate the role of miR396 in plant development and response to salt stress in this perennial monocot species. MiR396 TG plants display altered leaf morphology and tillering, and improved salt stress tolerance in comparison with wild-type (WT) controls. Further, genome-wide analysis in miR396 TG plants vs. WT controls elucidates the possible regulatory pathway for miR396-mediated salt or

other environmental stress responses, which provides insight into the central role of miR396 in the regulatory network.

3.2 Results

MiR396 is regulated by salt stress

Previous studies showed that miR396 responds to salt stress in various plant species, however, the expression profiles are different. For example, miR396 is down-regulated under salt stress in rice, *Spartina alternaiflora*, *Populus cathayana*, and *Salix matsudanabut* Koidz, but up-regulated in tomato (Chen, et al., 2015; Zhou, et al., 2012; Qin, et al., 2015; Gao, et al., 2010). To investigate the role of miR396 in response to salt stress in creeping bentgrass, we verify its response to salt stress through analyzing its expression profile. Stem-loop RT-qPCR analysis showed that miR396c was gradually up-regulated from 0 to 3 h salt treatment, and then declined at 6 h salt treatment but still elevated compared to 0 h control (Figure 3.1). The result suggests that miR396 might act as a positive regulator in plant salt stress response.

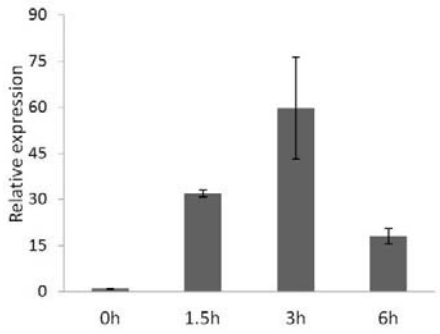

Figure 3.1 Expression profiles of miR396c in response to salt stress. WT creeping bentgrass plants were treated by applying 200 mM NaCl. Leaf samples at 0, 1.5, 3, and 6 h were collected for analyzing relative expression levels of mature miR396 under salt stress via stem-loop RT-qPCR. The relative changes in gene

expression were calculated based on the $2^{-\Delta\Delta CT}$ method. *UBIQUITIN5* (*AsUBQ5*) was used as reference gene. Data are presented as means of three technical replicates, and error bars represent ±SE.

Generation of transgenic creeping bentgrass constitutively expressing Osa-miR396c

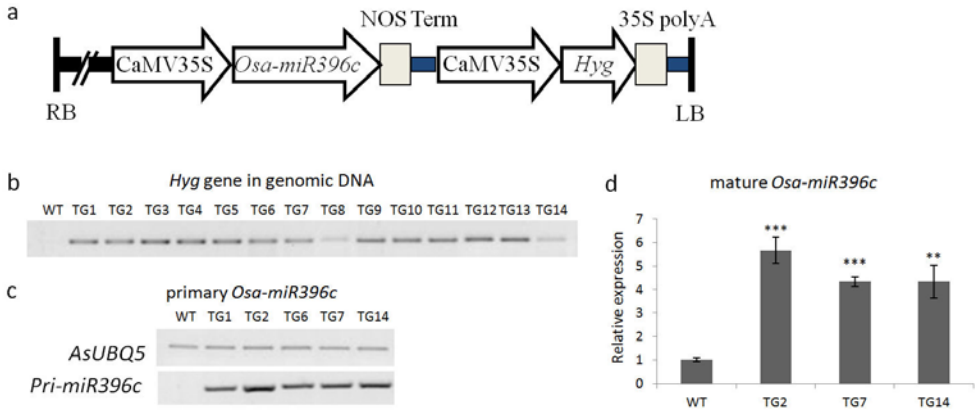

Figure 3.2 Generation of transgenic creeping bentgrass constitutively expressing *Osa-miR396c*. (a) *Osa-miR396c* gene constitutive expression construct of p35S-*Osa-miR396c*/p35S-*Hyg*. CaMV35S*:* Cauliflower Mosaic Virus 35S promoter. *Hyg*: the hygromycin resistance gene. RB: right border; LB: left border. (b) Examples of PCR analysis to amplify *Hyg* gene using genomic DNA of WT and TG plants. (c) Semi-quantitative RT-PCR analysis to compare the expression levels of primary *Osa-miR396c* in WT and TG plants. (d) Stem-loop RT-qPCR analysis to detect the expression of mature *Osa-miR396c* in TG and WT plants. The relative changes in gene expression were calculated based on the $2^{-\Delta\Delta CT}$ method. *AsUBQ5* was used as an endogenous control. Data are presented as means of three technical replicates, and error bars represent ±SE. Asterisks (** or ***) indicate a significant difference of expression levels between WT and each transgenic line at $P<0.01$ or 0.001 by Student's *t-test*.

After confirming that miR396c responds to salt stress, we generated transgenic creeping bentgrass constitutively expressing a rice gene, *Osa-miR396c* to further study the role of miR396 in plant adaption to salt stress (Figure 3.2a). The constitutive expression construct of miR396 was introduced into WT plants via *Agrobacterium tumefaciens*-mediated transformation. The selectable marker *Hyg* gene with hygromycin resistance was amplified from the genomic DNA of regenerated TG plants and WT controls for transgenic event selection (Figure 3.2b). Then the expression levels of *pri-miR396c* and mature *miR396c* were compared between WT and TG plants to determine

whether the rice pri-miR396c was successfully integrated into the genome of creeping bentgrass, transcribed, and properly processed (Figure 3.2c, d). We generated a total of 32 individual TG events, which exhibit two phenotypes, including coarse leaf (CL) and fine leaf (FL). In this study, TG2 (FL) and TG7 (CL) were used for further development analysis. Due to the extremely slow growth rate of FL lines, TG7 and TG14 (CL) were used for subsequent stress test.

Transgenic creeping bentgrass overexpressing miR396c exhibits altered plant development

To study if miR396 is implicated in the development of creeping bentgrass, 10-week old WT and TG plants each initiated from a single tiller were compared. As shown in Figure 3.3a, transgenic plants produced smaller shoots and fewer roots than those of WT controls. As a result, TG platns had significantly reduced biomass accumulation in both shoot and root compared to WT controls (Figure 3.3f, g). Further analysis of tiller growth indicates that the reduced shoot biomass in TG plants is associated with the shorter tiller length and/or low tiller number in comparison with WT controls (Figure 3.3c, h). The average length of the internodes from the longest transgenic tillers is significantly reduced compared with that of the WT tillers (Figure 3.3b, d, i), resulting in reduced tiller length in transgenics. When comparing leaf morphology between WT and TG plants, although transgenic leaves display CL and FL phenotypes, they are all narrower and shorter than WT leaves (Figure 3.3b, e, j, k), which is similar to the morphology changes observed in transgenic Arabidopsis and tobacco with constitutive expression of miR396

(Yang, et al., 2009b; Rodriguez, et al., 2010). The result suggests that the role of miR396 in controlling leaf development is conserved between monocots and dicots.

Previous study indicates that miR396 is involved in cell proliferation (Rodriguez, et al., 2010). To detect the impact of miR396 at cellular level, we cross-sectioned leaves and stems in WT and TG plants followed by histological analysis. As shown in Figure 3.4a and c, two transgenic lines have significantly fewer leaf veins than WT controls, resulting in narrower leaves. TG2 plants with FL phenotype displayed reduced stem diameter and a reduced number of vascular bundles, while TG7 plants with the CL phenotype did not show a significant difference from WT controls (Figure 3.4b, d, e). To investigate if the smaller leaf size in TG plants is attributed to the reduced cell proliferation, we compared the number of leaf epidermis cells in WT and TG plants. The result shows that the leaf epidermis cells in TG plants are significantly reduced compared to WT controls (Figure 3.4f, g), implying that miR396 is a negative regulator in cell proliferation.

MiR396 transgenic plants exhibit enhanced salt tolerance associated with improved water retention and cell membrane integrity, increased chlorophyll contents, and decreased proline contents under salt stress

To study the role of miR396 in plant response to salt stress in creeping bentgrass, we conducted salt stress treatment by applying 250 mM NaCl to WT and two transgenic lines, TG7 and TG14 for 8 days followed by a 10-day recovery. Before the stress test, WT and TG plants initiating from the same number of tillers were mowed to the same height. After NaCl treatment and during recovery, most of the WT leaves senesced and

turned yellow, while most of the transgenic leaves remained green with less damage (Figure 3.5a), indicating that overexpression of miR396c leads to enhanced salt tolerance in TG plants.

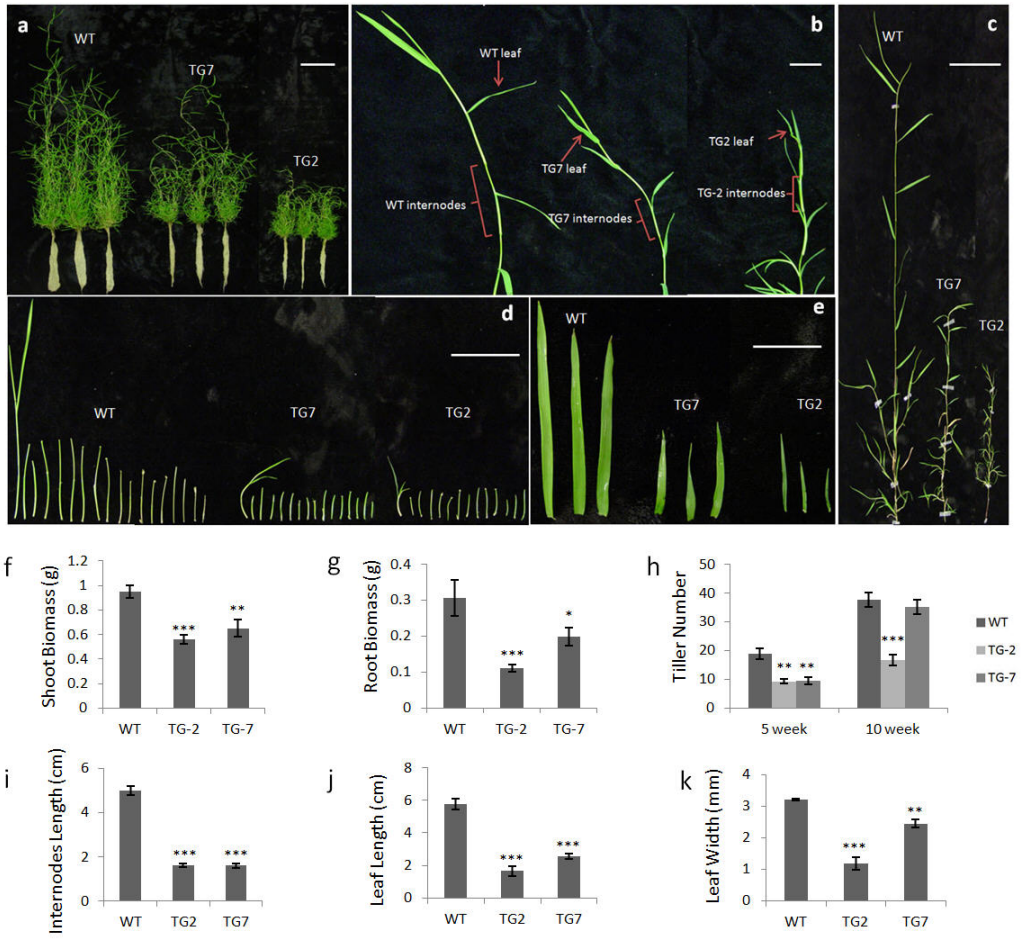

Figure 3.3 Plant tillering and development. (a) Ten-week-old WT and TG plants after initiation from a single tiller. Scale bar, 10 cm. (b) Representative leaves and internodes of WT and TG plants after initiation from single tillers. Scale bar, 2 cm. (c) Close up of the longest tillers from WT and TG plants. Scale bar, 5 cm. (d) All internodes from the representative longest tiller were sliced from top to bottom and arranged from left to right. Scale bar, 5 cm. (e) Three of the topmost fully developed leaves from the representative tillers of WT and TG plants. Scale bar, 2 cm. (f) Shoot and (g) root dry weight of WT and TG plants at 10 weeks after initiation from a single tiller (n=4). (h) Tiller number in WT and TG plants at five and ten weeks after initiation from a single tiller (n=5). (i) Average length of the topmost eight internodes from WT and TG tillers (n=6). (j) Leaf length and (k) leaf width from the representative WT and two transgenic lines (n=3). Data are showed as means, and error bars represent standard error. A significant difference between

WT and each transgenic line was indicated with asterisks (*, **, or ***) at $P<0.05$, 0.01, or 0.001 by Student's *t-test*.

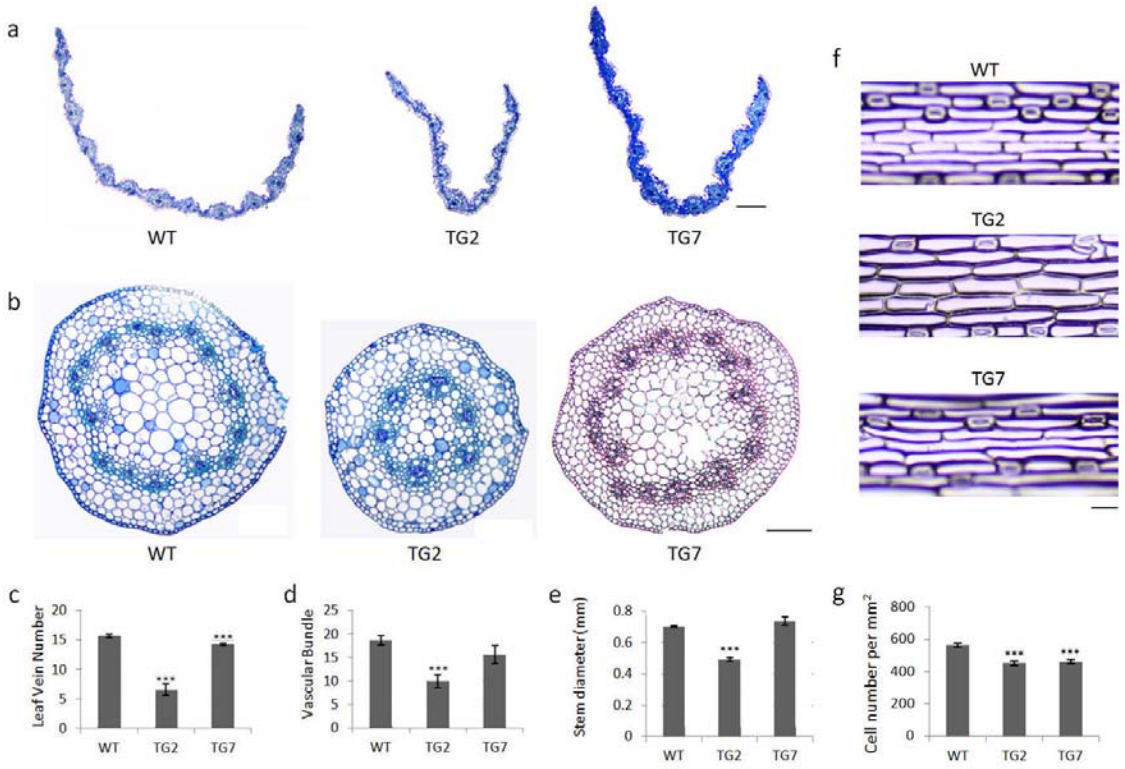

Figure 3.4 Histological analysis of leaf and stem of WT and TG plants. (a) Cross-section images of WT and TG leaves. Scale bar, 100 µm. (b) Cross-section images of WT and TG stems. Scale bar, 100 µm. (c) Statistical analysis of leaf vein number between representative WT and TG plants (n=5). (d) Statistical analysis of the number of vascular bundles between representative WT and TG stems (n=5). (e) Statistical analysis of stem diameter between WT and two transgenic lines (n=5). (f) The representative leaf epidermis of WT and two transgenic lines. Scale bar, 50 µm. (g) The number of leaf epidermis cells between WT and two transgenic lines (n=5). Data are showed as means with standard error. Asterisks (***) indicate a significant difference between WT and each transgenic line at $P<0.001$ by Student's *t-test*.

To further investigate what causes the enhanced salt tolerance in TG plants at physiological level, we analyzed leaf relative water content (RWC), leaf electrolyte leakage (EL), total chlorophyll content, and proline content in WT and TG plants before and 8 days after 250 mM NaCl treatment. Before the treatment, WT plants and two transgenic lines have similar RWC (Figure 3.5b). Upon exposure to salt stress, although

the RWC of both WT and TG plants declined, the decline in TG plants was significantly less pronounced than that of WT controls (Figure 3.5b), indicating that TG plants have a better capacity of water retention under salt stress. The EL of WT and TG plants was similar under normal growth conditions, and then increased dramatically after NaCl treatment (Figure 3.5c). However, EL in TG plants was significantly lower than that in WT controls (Figure 3.5c), suggesting that TG plants have improved ability to maintain cell membrane integrity during salt exposure. Salt stress leads to chlorophyll breakdown and induces leaf yellowing. In this study, total chlorophyll content of WT and TG plants was similar before the salt stress test, but declined after salt stress exposure (Figure 3.5d). However, the decline in WT controls was significantly more pronounced than that in TG plants (Figure 3.5d), which may contribute to the robustness of photosynthetic system. Proline is accumulated under salinity conditions and acts as an osmolyte to protect plants from salt stress-induced toxic oxygen derivatives. Proline contents in WT and miR396 TG creeping bentgrass plants were similar under normal growth conditions, but increased dramatically after NaCl application (Figure 3.5e). Interestingly, proline content in two transgenic lines was significantly lower than that in WT controls (Figure 3.5e). It is likely that the less salt-elicited leaf damage in TG plants triggers less accumulation of proline than WT controls.

Enhanced salt tolerance in TG plants is associated with reduced Na^+ uptake

The adaptive mechanisms of plants in response to salt stress include exclusion of Na^+ and/or compartmentalization of Na^+ into the vacuole. To elucidate the mechanism of

miR396-mediated salt tolerance, we compared the Na^+ content between WT and TG plants under normal and salt stress conditions. The result shows that the two transgenic lines accumulate significantly less Na^+ than WT contols under salt stress conditions (Figure 3.6a), suggesting that the enhanced salt tolerance in miR396 transgenics may be attributed to the exclusion of Na^+.

High concentration of Na^+ during salt stress leads to limited K^+ uptake and it might substitute for K^+ in some K^+-dependent protein interactions within plant cells. Thus, the ability of K^+ retention and a high K^+: Na^+ ratio is important for salt tolerance. In this study, the K^+ relative content is similar in WT and TG plants under both normal and stressed conditions (Figure 3.6b), while the K^+: Na^+ ratio is significantly induced in TG plants under salinity conditions (Figure 3.6c), which might contribute to the enhanced salt tolerance.

MiR396 is involved in the regulation of Na^+ transporter SOS1

The relatively low Na^+ content in TG plants under salt stress conditions might result from the enhanced ability to extrude Na^+ from the cytosol across the plasma membrane. SOS1 is the best-known Na^+ transporter, which functions in Na^+ exclusion. To reveal the molecular mechanism of miR396-mediated salt tolerance, we cloned a partial sequence of *SOS1* in creeping bentgrass and analyzed the expression levels of *AsSOS1* in WT and two transgenic lines. RT-PCR analysis shows that *AsSOS1* exhibits elevated expression in TG plants compared with WT controls (Figure 3.7), suggesting that miR396 mediates salt tolerance through Na^+ excluding mechanism.

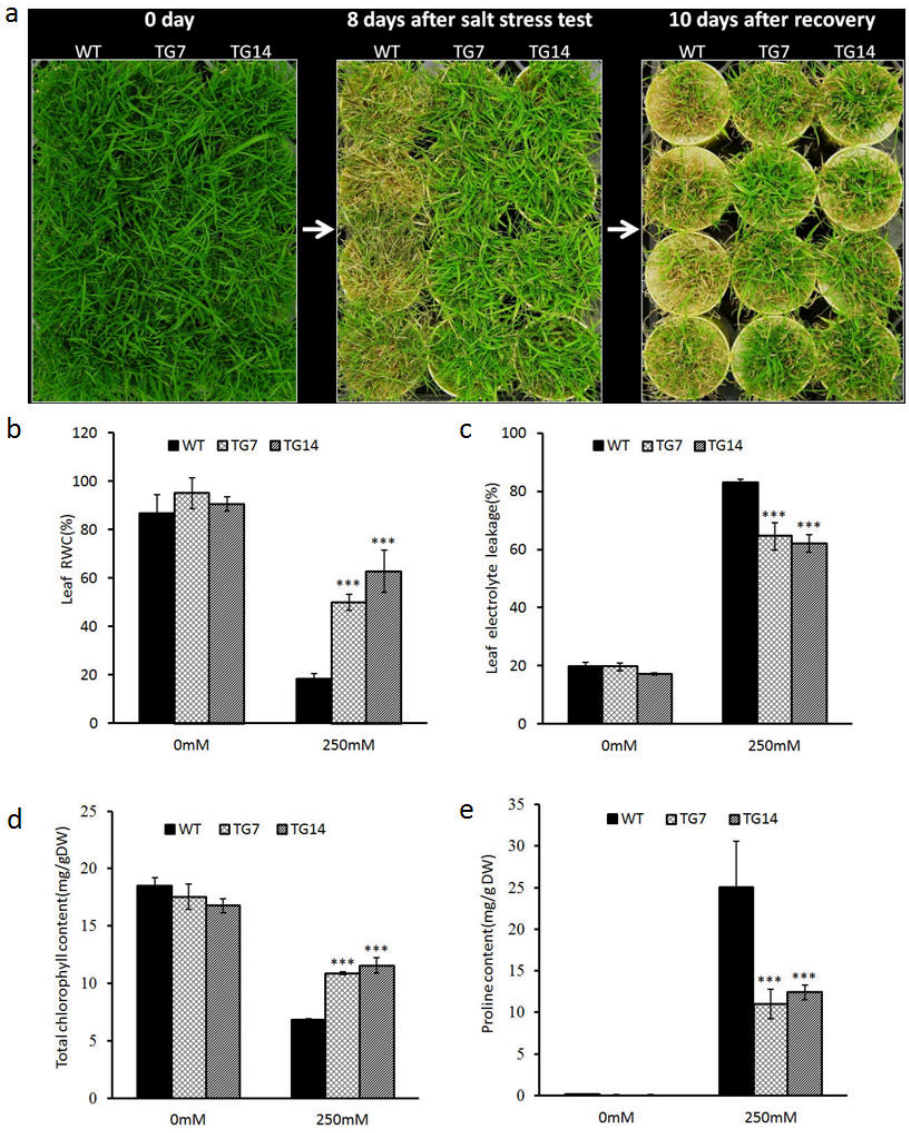

Figure 3.5 Responses of WT plants and transgenic lines under salt stress test. (a) WT and TG plants were subjected to 250 mM NaCl treatment for 8 days followed by a 10-day recovery. (b) Leaf RWC, (c) leaf electrolyte leak, (d) total chlorophyll content, and (e) proline content were measured in WT and TG leaves at 0 day and 8 days of salt stress treatment. DW, dry weight. Data are showed as means (n=4) with standard error. Asterisks (***) indicate a significant difference between WT and each transgenic line at $P<0.001$ by Student's *t-test*.

Identification of miR396c putative targets and their responses to salt stress

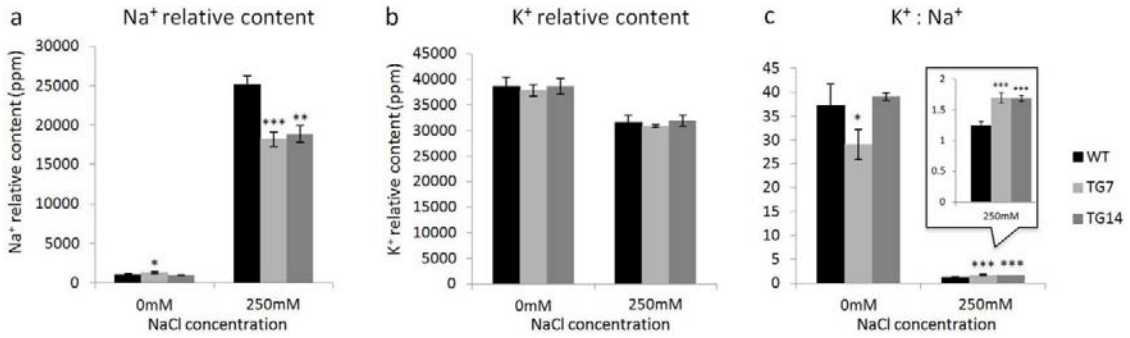

Figure 3.6 Na^+ and K^+ relative contents. (a) Na^+ relative content and (b) K^+ relative content were measured before and after salt stress treatment in the leaf tissue of WT and two transgenic lines. (c) K^+: Na^+ ratio before and after salt test in WT and two transgenic events. Data are showed as means (n=3) with standard deviation. Asterisk(s) indicates significant differences between WT and each TG line at *, P<0.05; **, P<0.01; ***, P< 0.001 by Student's *t-test*.

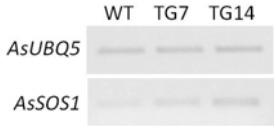

Figure 3.7 Semi-quantitative RT-PCR analysis of the expression levels of *AsSOS1* in WT and two transgenic lines under normal growth conditions. *AsUBQ5* was used as the reference gene.

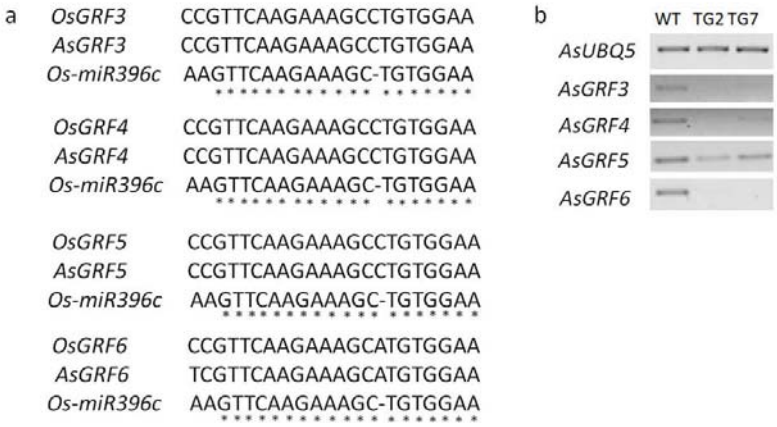

Figure 3.8 Identification of putative miR396c targets in creeping bentgrass. (a) A comparison of miR396c target sites in the putative targets *AsGRF3, AsGRF4, AsGRF5,* and *AsGRF6* between rice andcreeping bentgrass. Asterisks indicate the identical RNA sequences. (b) Semi-quantitative RT-PCR

analysis of *AsGRF3, AsGRF4, AsGRF5,* and *AsGRF6* expression in WT and TG plants. *AsUBQ5* was used as an endogenous control.

To elucidate the molecular mechanism of miR396-mediated plant development and salt tolerance, we sought to identify the putative targets of miR396 in creeping bentgrass. It has been reported that the targets of miR396 encode the transcription factors of the growth regulating factor (GRF) family. In Arabidopsis, *GRF* gene family contains nine members (*AtGRF1-9*), seven of which have miR396 binding site except *AtGRF5* and *AtGRF6* (Debernardi, et al., 2012). In rice, twelve members (*OsGRF1-12*) were identified, ten of which contain miR396c target site except *OsGRF11* and *OsGRF12* (Debernardi, et al., 2012). Expression analysis of *OsGRFs* in WT rice and transgenic rice overexpressing *Osa-miR396d* indicates that nine out of twelve *OsGRFs* are repressed in TG plants, which are *OsGRF1-8* and *OsGRF10* (Liu, et al., 2014). In this study, four *GRFs* (*AsGRF3-6*) were successfully cloned in creeping bentgrass based on the sequence of *GRF* homologs in rice and Brachypodium. MiR396c target sites in *AsGRF3-6* were identified and compared with the target sites of rice *OsGRF3-6* as shown in Figure 3.8a. The expression of these four genes was repressed in TG2 and TG7 lines compared with that in WT controls (Figure 3.8b), suggesting that they are the putative targets of miR396c in creeping bentgrass. GRFs play an essential role in plant leaf, stem, and root growth and development (Omidbakhshfard, et al., 2015). Therefore, is is plausible to speculate that the repressed expression of the *AsGRFs* might contribute to the altered plant development in miR396 TG creeping bentgrass.

Next, we analyzed the expression patterns of *AsGRFs* under salt conditions to investigate how the miR396-GRF pathway is implicated in plant salt tolerance. Semi-quantitative RT-PCR analysis shows that expression levels of *AsGRF3-6* are all up-regulated in response to 250 mM NaCl treatment (Figure 3.9).

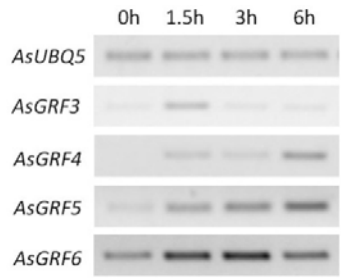

Figure 3.9 Expression profiles of miR396c putative targets in response to salt stress. Transcript levels of *AsGRF3*, *AsGRF4*, *AsGRF5*, and *AsGRF6* were analyzed under 0, 1.5, 3, and 6 h after 250 mM NaCl treatment using semi-quantitative RT-PCR. *AsUBQ5* was used as an endogenous control.

Genome-wide analysis of miR396-mediated plant development and salt stress tolerance

MiRNAs serve as master regulators to integrate different regulatory pathways to control plant development and cope with abiotic stress. To gain insight into the miR396-mediated regulatory network, we performed RNA-seq analysis to study the differentially expressed genes (DEGs) in miR396 TG plants vs. WT controls. Equal mixtures of RNA isolated from leaves and shoot apical meristems of non-stressed WT and TG plants were used for cDNA library preparation. Illumina sequencing generated 4,444,691 contigs, which were further assembled into 82,819 unigenes with an average size of 995.5 bp. The reproducibility of RNA-seq analysis was confirmed by multidimensional scaling (MDS) plot (Figure A-7a), which shows the expected consistency between two biological

replicates of WT and TG samples, respectively. A volcano plot shows the distribution of Log_2 fold changes (FC) of 17,338 unigenes at false discovery rate (FDR) corrected *p* value < 0.05 (Figure A-7b, c). Among the differentially expressed transcripts (Log_2 FC > 2 or < -2), 584 are up-regulated and 1027 are down-regulated in TG plants vs. WT controls.

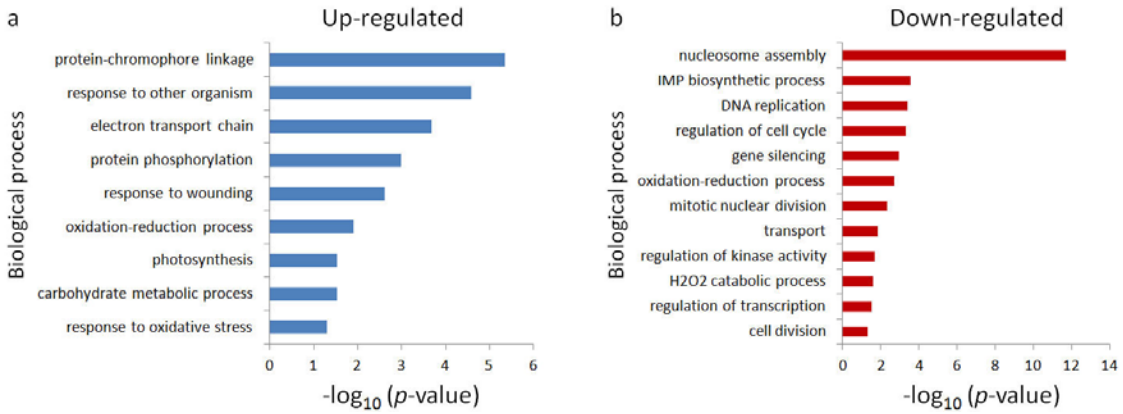

Figure 3.10 GO enrichment analysis. Significantly enriched GO terms show biological processes of (a) up-regulated (log_2 FC > 2, over-represented *p*-value < 0.05) and (b) down-regulated (log_2 FC < -2, over-represented *p*-value < 0.05) transcripts in TG vs. WT.

Gene ontology enrichment analysis

To identify the putative biological processes of DEGs in TG vs. WT plants, we performed gene ontology (GO) enrichment analysis. In this study, 9 and 12 GO terms in the biological process category were significantly enriched (over-represented *p*-value <0.05) in the up- and down-regulated genes (log_2 FC > 2 or log_2 FC < -2), respectively (Figure 3.10). Among them, significantly enriched GO terms of 'oxidation-reduction process', 'response to oxidative stress', and 'hydrogen peroxide catabolic process' suggest that miR396 might be involved in the oxidation-reduction process. Salt stress

results in ROS accumulation in plant cells and creates oxidative environment and imbalanced redox state. Oxidation-reduction process plays a fundamental role in regulating redox homeostasis and might contribute to the enhanced salt tolerance in miR396 TG plants. Besides the stress tolerance-related GO terms, GO terms of 'DNA replication', 'cell division', 'regulation of cell cycle', and 'mitotic nuclear division' were all significantly enriched in the down-regulated genes (Figure 3.10b), indicating that TG plants might have less cell division and fewer cell numbers, which is consistent with what we observe in transgenic leaves.

Differential expression of leaf development- and stress response-related genes

In addition to identify the overrepresented GO terms in the whole DEGs of TG vs. WT dataset, we also analyzed the leaf development- and stress response-related DEGs. Significantly enriched GO terms (over-represented p-value < 0.05) in the DEGs ($\log_2$ FC > 1 or $\log_2$ FC < -1, FDR corrected p-value < 0.05) were identified, in which the corresponding genes related to leaf development and environmental stress response were chosen for generating a heatmap. As shown in Figure 3.11, 201 DEGs had enriched GO terms of 'DNA replication', 'cell differentiation', 'cell division', 'leaf development', *etc*. In addition, 603 DEGs had enriched GO terms related to biotic and abiotic stress responses, including response to cold, salt, heat, drought, wounding, light, heavy metal, nutrient deficiency, bacterium, virus, and fungus (Figure 3.11). The result demonstrates that miR396 affects a variety of biological processes.

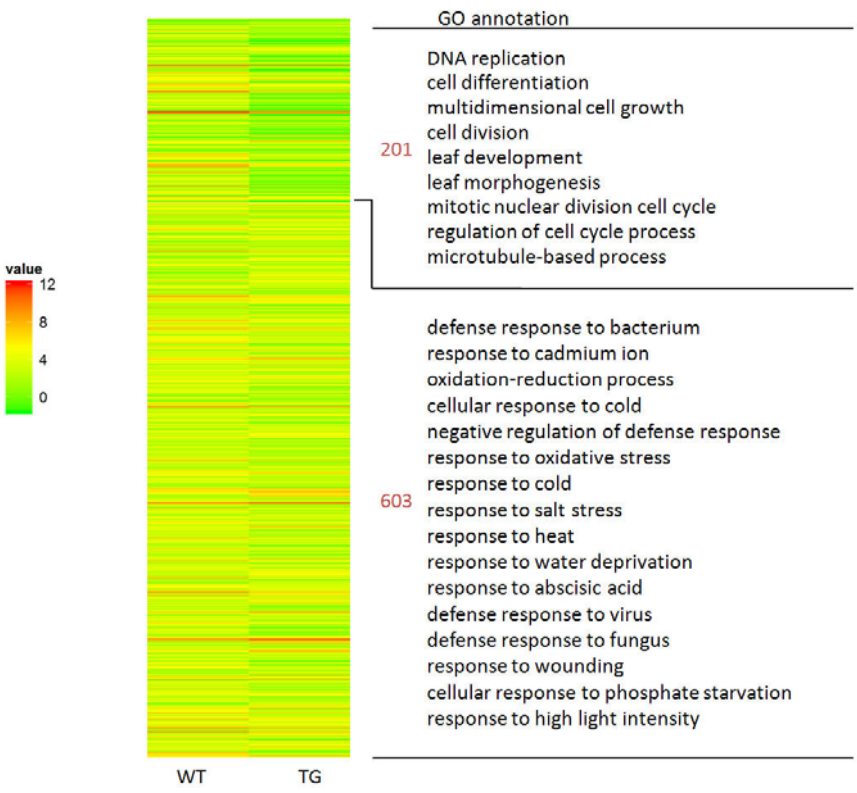

Figure 3.11 Differential expression of leaf development- and environmental stress response-related transcripts. Significantly enriched genes (over-represented p-value < 0.05) from each group were selected to generate the heatmap. Right to the heatmap shows the number of significantly enriched genes and GO terms. The color gradient shows the $\log_2$-transformation of the read count value.

Validation of expression profiles of candidate genes

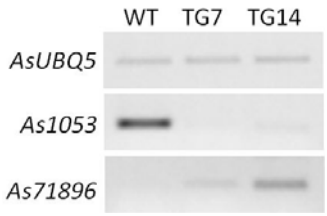

Figure 3.12 Semi-quantitative RT-PCR analysis of the expression patterns of DEGs *As1053* and *As71896* in WT and transgenic (TG7 and TG14) plants under normal growth conditions. *AsUBQ5* was used as an endogens control.

To validate the expression profiles of DEGs in RNA-seq data, we analyzed expression levels of candidate genes involved in cell reproduction and environmental stress response. *As1053* is predicted to encode a subunit of anaphase-promoting complex involved in cell reproduction. RT-PCR analysis shows repressed expression in two transgenic lines compared with that in WT controls (Figure 3.12), which is in agreement with RNA-seq result. *As71896* is predicted to be a WRKY40 transcription factor gene. Its transcription is up-regulated in TG plants in comparison with WT controls (Figure 3.12), which is consistent with the RNA-seq data.

3.3 Discussion

MiR396-mediated plant development during vegetative growth

The *miR396-GRF* regulatory pathway plays a key role in leaf development. Overexpression of *At-miR396a* leads to smaller and narrower leaves through supressing *GRF* genes in transgenic tobacco (Yang, et al., 2009a). In this study, transgenic creeping bentgrass overexpressing *Os-miR396c* resulted in similar leaf phenotype and down-regulated *AsGRFs*, implying that *miR396-GRF* pathway is functionally conserved in different plant species.

The role of GRFs in regulating leaf development has been well characterized in Arabidopsis. The quadruple mutant *atgrf1/2/3/4* exhibits smaller leaf size because of the reduced cell size and cell number (Omidbakhshfard, et al., 2015). *AtGRF1* and *AtGRF2* control cell expansion, while *AtGRF3* and *AtGRF4* regulate cell proliferation (Omidbakhshfard, et al., 2015). In this study, we compared the number of leaf epidermis

cells per unit leaf area between WT and TG plants. The result shows that TG plants have significantly fewer leaf epidermis cells per unit area than that of WT controls, which also implies larger cell size in TG plants. Additionally, RT-PCR analysis shows that *As1053*, a putative gene encoding anaphase-promoting complex subunit which is involved in cell proliferation, is down-regulated in miR396 TG plants. Thus, it is likely that *miR396c*/*GRF* module control leaf area through regulating cell proliferation instead of cell expansion. This is further supported by the GO enrichment analysis, in which the GO terms of 'DNA replication', 'cell division', 'regulation of cell cycle', and 'mitotic nuclear division' were significantly enriched in the down-regulated genes.

In grasses, tillering plays an essential role in shoot architecture and grain yield. In this study, tillering differences were observed between WT and miR396 TG creeping bentgrass. TG plants accumulate less shoot biomass, and display fewer tillers, and reduced tiller length due to shorter internodes, suggesting that miR396 is implicated in the regulation of tillering. The development of grass tillers is determined by shoot apical meristems (SAMs), since all aerial organs are produced from SAMs. Previous studies show that *KNOTTED1-LIKE HOMEOBOX* (*KNOX*) transcription factor genes are key controllers on SAM formation and maintenance (Long, et al., 1996; Vollbrecht, et al., 2000). Recently, GRFs have been reported to regulate the expression of KNOX family gene *OsKN2* in rice through interacting with the promoter of *OsKN2* (Kuijt, et al., 2014). The similar interactions between GRF and *KNOX* have also been reported in Arabidopsis and barley (Kuijt, et al., 2014; Osnato, et al., 2010). Therefore, we speculate that the

altered tillering in TG creeping bentgrass is mediated through the *miR396-GRF-KNOX* regulatory module.

MiR396 is also involved in the modulation of root development. Transgenic *Medicago truncatula* overexpressing miR396b exhibits reduced root dry weight, while MIM396, the inactive form of miR396 transgenic line displays significantly increased root dry weight compared with WT controls (Bazin, et al., 2013). Further analysis indicates that miR396 modulates root growth through restricting the activity of root cell division (Bazin, et al., 2013). In this study, the phenotype of reduced root dry weight is also observed in miR396 transgenic creeping bentgrass, indicating the conserved function of miR396-mediated root development in both monocots and dicots.

MiR396-mediated regulatory network in plant response to salt stress

Plant responses to high salinity conditions have been extensively studied at the molecular level. The products of high salinity-responsive genes include downstream functional proteins and upstream regulatory proteins. In this study, *AsSOS1*, encoding the downstream functional Na^+/H^+ antiporter, was up-regulated in miR396 TG plants. Under salinity stress, SOS1 functions in maintaining ion homeostasis. Previous studies show that overexpression of *SOS1* leads to enhanced salt tolerance in transgenic Arabidopsis, tobacco, and *Chrysanthemum crassum* (An, et al., 2014; Yang, et al., 2009b; Yue, et al., 2012). It is plausible that the enhanced salt tolerance in transgenic creeping bentgrass result from miR396-mediated positive regulation of *AsSOS1*. Besides ionic stress, high salinity leads to the production of ROS, which damages plant cells and tissues through

interacting with key macromolecules and metabolites. Among functional proteins, antioxidant enzymes protect plants from oxidative stress caused by ROS accumulation. In this study, ignificantly enriched GO terms of 'oxidation-reduction process', 'H_2O_2 catabolic process', 'response to oxidative stress', 'oxidoreductase activity', and 'peroxidase activity' imply that miR396 is crucial in plant response to oxidative stress.

Upstream regulatory proteins involved in high salinity responses include transcription factors, protein kinases, and phosphatases. The GO enrichment analysis shows that 'regulation of transcription' and 'regulation of kinase activity' are significantly enriched in the down-regulated genes (Figure 3.10b). Further RT-PCR analysis shows that TF gene *WRKY40* is induced in miR396 TG plants. TFs from WRKY family positively regulate salt and other environmental stress tolerance in a variety of plant species (Wang, et al., 2015; Zhao, et al., 2015). In addition to salt stress response, significantly enriched DEGs also participate in the biological processes of response to heat, cold, drought, heavy metal, wounding, high light intensity, nutrient deficiency stresses, and defense response to bacterium, virus, and fungus (Figure 3.12). Therefore, the result indicates that miR396, in a broad sense, is actively involved in multiple environmental stress responses through modulating both functional and regulatory proteins.

MiR396 exerts its function at post-transcriptional and post-translational levels to regulate its targets, which encode TFs of GRF family and some species-specific TFs (e.g. bHLH79 in Medicago, SVP in Arabidopsis) (Bazin, et al., 2013; Yang, et al., 2015). These TFs will further regulate their direct targets, including TFs or other regulatory and

functional proteins (e.g. AtGRF7 targets TF gene *DREB2A* in Arabidopsis, OsGRF3 and OsGRF10 target TF gene *OsKN2* in rice, OsGRF6 and OsGRF10 target protein kinase gene *OsCR4* and demethylase gene *OsJMJ706*) (Kim, et al., 2012; Kuijt, et al., 2014; Liu, et al., 2014). Therefore, miR396 serves as an important master regulator to integrate various regulatory elements to cope with salt and other environmental stresses.

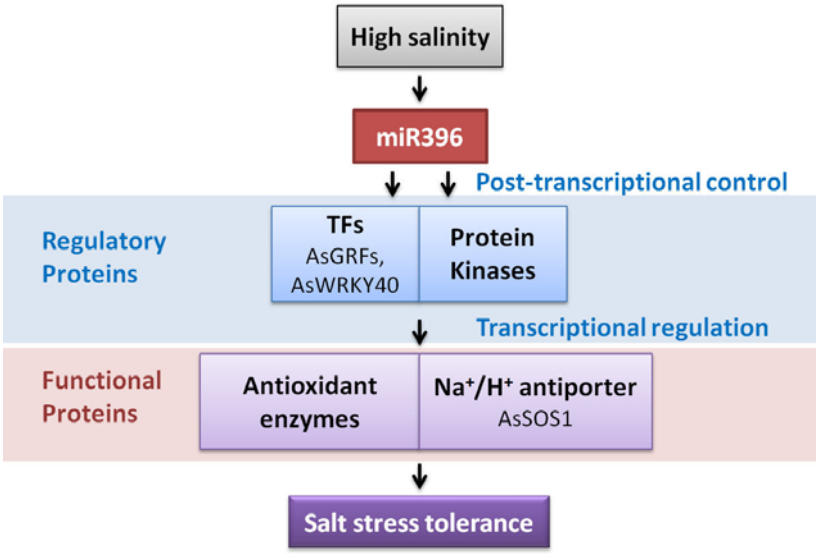

Figure 3.13 Hypothetical model of the signaling pathway of miR396-mediated salt stress tolerance.

In this study, a signaling pathway of miR396-mediated salt tolerance in creeping bentgrass was proposed (Figure 3.13). In response to high salinity, miR396 is induced to post-transcriptionally control the regulatory proteins of protein kinases and TFs, such as GRFs and WRKY40. Next, these regulatory proteins activate downstream salinity responsive genes, such as Na^+/H^+ antiporter *AsSOS1* and detoxification enzyme genes to protect plant cells from ionic stress and oxidative stress causd by high salinity stress. This study provides insight into miR396-mediated salt tolerance and allows deciphering the

role of miRNAs in the complex regulatory network in plant response to environmental stress.

3.4 Materials and methods

Generation of transgenics overexpressing miR396

A 510 bp DNA fragment of *Os-miR396c* gene (GenBank: AK062523.1) containing pre-miR396c stem-loop structure was amplified from rice cDNA. The forward and reverse primer set was 5'-TCTAGATTTTAACCCATCCAATGCCC-3' containing an XbaI recognition site (underlined) and 5'-GTCGACCTCTCTCTCTCTCTCTGCCTG-3' containing a SalI recognition site (underlined). The cDNAs were then cloned into the pGEM-T Easy vector (Promega, Madison, WI). T Easy-cDNA with the correct sequence was digested and recombined into the binary vector pZH01 generating an overexpression gene construct, which was then introduced into creeping bentgrass cultivar 'Penn A-4' (supplied by HybriGene) via *Agrobacterium tumefaciens* strain LBA4404-mediated plant transformation. Creeping bentgrass transformation using mature seed-initiated embryonic callus was as described previously (Luo, et al., 2004a; Luo, et al., 2004b).

Plant growth and salt stress treatment

WT and the regenerated TG creeping bentgrass were clonally propagated and were grown in cone-tainers (4.0 × 20.3 cm, Dillen Products) filled with pure silicon sand. The plants were fertilized every other day with 0.2g/L 20:10:20 water-soluble fertilizer (Peat-Lite Special; The Scotts Company) and maintained in a growth room with short day light regime (14-h of light/ 10-h of dark). Temperatures in the short day growth room were

25°C during the light period and 17°C during the dark period with 350-450 μmol m^{-2} s^{-1} light intensity. The conditions for the long day growth room were the same as the short day growth room, except that the light regime is 16 h of light/8 h of dark. WT and TG plants were propagated at the same time and from the same amount of tillers to ensure that they were at the same developmental stage for morphological analysis and salt stress treatment.

Salt stress treatment and leaf sample collection for gene expression analysis were performed as described previously with modifications (Yuan, et al., 2015). In this study, the concentration of NaCl solution was 250 mM. The duration for salt stress treatment was 8 days followed by a 10-day recovery.

Measurement of mineral content, leaf RWC, chlorophyll, and proline content

WT and TG plant leaf tissues were collected before and after 8-day, 250 mM NaCl treatments for the measurement of Na^+ and K^+ relative contents, leaf RWC, EL, total chlorophyll content, and proline content using previously published protocols (Haynes, 1980; Bates, et al., 1973; Li, et al., 2010).

Histology Analysis

Histological analysis of the leaf and stem cross sections was performed as described previously with modifications (Yuan, et al., 2015). In this study, sections were stained using Toluidine Blue or Safranin. For leaf epidermis cells observation, clear nail polish was applied to the leaf upper epidermis of the representative WT and TG plants. After

drying completely, the films were peeled off and observed under microscope (MEIJI EMZ-5TR).

Plant RNA isolation and expression analysis

Plant total RNA was isolated from 100 mg of the 1st and 2nd topmost fully expanded leaves of each tiller. RNA isolation, semi-quantitative RT-PCR and stem-loop RT-qPCR analyses were performed according to previously published protocol (Yuan, et al., 2015). *AsUBQ5* (JX570760) was used as an endogenous control. The miR396c stem-loop RT primer, PCR forward and reverse primers are 5'-GTCTCCTCTGGTGCAGG GTCCGAGGTATTCGCACCAGAGGAGACAAGTTC-3', 5'-GCGGTTCCACAGCTT TCTT-3', and 5'-TGGTGCAGGGTCCGAGGTATT-3', respectively.

cDNA library preparation and Illumina sequencing

Total RNA of LD 3-week leaves and SAMs from nonvernalized WT and TG plants were isolated and purified using the RNeasy Plant Mini Kit (Qiagen, Germantown, MD). RNA quality was evaluated by the measurement of A260/A280 and A260/A230 ratios. Only RNA samples with an A260/A280 ratio greater than 1.8 and an A260/A230 ratio greater than 2.0 were used. For complete gene expression information, an equal mixture of RNA samples from leaves and SAMs was used for the construction of cDNA libraries using TruSeq RNA Library Preparation v2 (Illumina Inc., San Diego, CA) according to the manufacturer's protocol. Paired-end sequencing of each library was performed using the HiSeq 2000 (Illumina Technologies) platform following the manufacturer's instructions (101-bp paired-end reads). The raw reads (range from 12.5 to 13.8 million

for each of the two replicates of WT and TG) were evaluated for quality using FastQC 11.3 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and then were trimmed using Trimmomatic 0.32 (Bolger, et al., 2014). The trimmed reads (range from 12.1 to 13.2 million) from four cDNA libraries (two replicates for both WT and TG) were used to generate a *de novo* assembly using Trinity, starting with in silico read normalization followed by TrinityRNASeq (version: trinityrnaseq_r2014-04-13) with default *k*-mer length of 25 (Grabherr, et al., 2011). The Trinity output contained 4,444,691 contigs. To remove redundancy and unlikely transcripts the pipeline followed with TransDecoder (http://transdecoder.github.io/), a minimum protein length of 50, and CD-HIT (Li and Godzik, 2006; Fu, et al., 2012). A final reference unigene set containing 82,819 sequences was used for further data analysis.

Differential expression analysis

To estimate the expression levels, reads from each sample were aligned to the final reference unigene set using Bowtie2 and TopHat (Langmead and Salzberg, 2012; Trapnell, et al., 2009). A maximum of two mismatches were allowed for the alignments. Counts for each gene were generated by Subread's featureCount software (Liao, et al., 2014). The differential gene expression analysis was performed using R 3.2.0 (https://www.r-project.org) and the Bioconductor package edgeR with FDR corrected *p*-value cut-off of < 0.05 (Robinson, et al., 2010). A MDS plot was generated using edgeR to show the separation between WT and TG samples and consistency between replicates. A volcano plot was created using edgeR to plot $\log_2$ FC and the $-\log_{10}$ *p*-value in TG vs

WT. The heatmaps showing expression profiles between WT and TG samples were generated based on the log_2-transformed count values using R's pHeatmap.2 package (https://cran.r-project.org/web/packages/pheatmap/index.html).

GO enrichment analysis

To gain information on the over-represented functional categories, GO enrichment analysis was performed. Since there is no GO annotation available for creeping bentgrass transcripts, putative GO terms were assigned using NCBI-blast+ and Blast2GO 2.8 (Conesa, et al., 2005; Camacho, et al., 2009). GO enrichment of WT and TG data sets was performed using Bioconductor's goseq package with over-represented *p*-value cut-off of < 0.05 (Young, et al., 2010). GO categories containing less than or equal to three genes were filtered out.

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References

Agarwal PK, Shukla PS, Gupta K, Jha B (2013) Bioengineering for salinity tolerance in plants: state of the art. Mol Biotechnol **54:** 102-123

An J, Song A, Guan Z, Jiang J, Chen F, Lou W, Fang W, Liu Z, Chen S (2014) The over-expression of *Chrysanthemum crassum* CcSOS1 improves the salinity tolerance of chrysanthemum. Mol Biol Rep **41:** 4155-4162

Apse MP, Blumwald E (2007) Na transport in plants. FEBS Lett **581:** 2247-2254

Apse MP, Aharon GS, Snedden WA, Blumwald E (1999) Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in Arabidopsis. Science **285:** 1256-1258

Bates L, Waldren R, Teare I (1973) Rapid determination of free proline for water-stress studies. Plant Soil **39:** 205-207

Baucher M, Moussawi J, Vandeputte OM, Monteyne D, Mol A, Pérez-Morga D, El Jaziri M (2013) A role for the miR396/GRF network in specification of organ type during flower development, as supported by ectopic expression of Populus trichocarpa miR396c in transgenic tobacco. Plant Biol **15:** 892-898

Bazin J, Khan GA, Combier J, Bustos-Sanmamed P, Debernardi JM, Rodriguez R, Sorin C, Palatnik J, Hartmann C, Crespi M (2013) miR396 affects mycorrhization and root meristem activity in the legume *Medicago truncatula*. Plant J **74:** 920-934

Ben Ahmed C, Ben Rouina B, Sensoy S, Boukhriss M, Ben Abdullah F (2010) Exogenous proline effects on photosynthetic performance and antioxidant defense system of young olive tree. J Agric Food Chem **58:** 4216-4222

Blumwald E (2000) Sodium transport and salt tolerance in plants. Curr Opin Cell Biol **12:** 431-434

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics **30:** 2114-2120

Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL (2009) BLAST+: architecture and applications. BMC Bioinformatics **10:** 421-2105-10-421

Casadevall R, Rodriguez RE, Debernardi JM, Palatnik JF, Casati P (2013) Repression of growth regulating factors by the microRNA396 inhibits cell proliferation by UV-B radiation in Arabidopsis leaves. Plant Cell **25:** 3570-3583

Chen L, Luan Y, Zhai J (2015) Sp-miR396a-5p acts as a stress-responsive genes regulator by conferring tolerance to abiotic stresses and susceptibility to *Phytophthora nicotianae* infection in transgenic tobacco. Plant Cell Rep 1-13

Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, Robles M (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics **21:** 3674-3676

Debernardi JM, Mecchia MA, Vercruyssen L, Smaczniak C, Kaufmann K, Inze D, Rodriguez RE, Palatnik JF (2014) Post-transcriptional control of GRF transcription factors by microRNA miR396 and GIF co-activator affects leaf size and longevity. Plant J **79:** 413-426

Debernardi JM, Rodriguez RE, Mecchia MA, Palatnik JF (2012) Functional specialization of the plant miR396 regulatory network through distinct microRNA–target interactions. PLoS Genet **8**: e1002419

Eltayeb AE, Kawano N, Badawi GH, Kaminaka H, Sanekata T, Shibahara T, Inanaga S, Tanaka K (2007) Overexpression of monodehydroascorbate reductase in transgenic tobacco confers enhanced tolerance to ozone, salt and polyethylene glycol stresses. Planta **225:** 1255-1264

Fu L, Niu B, Zhu Z, Wu S, Li W (2012) CD-HIT: accelerated for clustering the next-generation sequencing data. Bioinformatics **28:** 3150-3152

Gao P, Bai X, Yang L, Lv D, Li Y, Cai H, Ji W, Guo D, Zhu Y (2010) Over-expression of osa-MIR396c decreases salt and alkali stress tolerance. Planta **231:** 991-1001

Garg AK, Kim JK, Owens TG, Ranwala AP, Choi YD, Kochian LV, Wu RJ (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc Natl Acad Sci USA **99:** 15898-15903

Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol **29:** 644-652

Guan Z, Chai T, Zhang Y, Xu J, Wei W (2009) Enhancement of Cd tolerance in transgenic tobacco plants overexpressing a Cd-induced catalase cDNA. Chemosphere **76:** 623-630

Gupta AS, Heinen JL, Holaday AS, Burke JJ, Allen RD (1993) Increased resistance to oxidative stress in transgenic plants that overexpress chloroplastic Cu/Zn superoxide dismutase. Proc Natl Acad Sci USA **90:** 1629-1633

Hasegawa PM, Bressan RA, Zhu J, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. Annu Rev Plant Physiol Plant Mol Biol **51:** 463-499

Hauser F, Horie T (2010) A conserved primary salt tolerance mechanism mediated by HKT transporters: a mechanism for sodium exclusion and maintenance of high K^+ /Na^+ ratio in leaves during salinity stress. Plant, Cell Environ **33:** 552-565

Haynes R (1980) A comparison of two modified Kjeldahl digestion techniques for multi-element plant analysis with conventional wet and dry ashing methods. Commun Soil Sci Plan **11:** 459-467

Kim JH, Choi D, Kende H (2003) The AtGRF family of putative transcription factors is involved in leaf and cotyledon growth in Arabidopsis. Plant J **36:** 94-104

Kim JS, Mizoi J, Kidokoro S, Maruyama K, Nakajima J, Nakashima K, Mitsuda N, Takiguchi Y, Ohme-Takagi M, Kondou Y, Yoshizumi T, Matsui M, Shinozaki K, Yamaguchi-Shinozaki K (2012)

Arabidopsis growth-regulating factor7 functions as a transcriptional repressor of abscisic acid- and osmotic stress-responsive genes, including DREB2A. Plant Cell **24:** 3393-3405

Kuijt SJ, Greco R, Agalou A, Shao J, 't Hoen CC, Overnas E, Osnato M, Curiale S, Meynard D, van Gulik R, de Faria Maraschin S, Atallah M, de Kam RJ, Lamers GE, Guiderdoni E, Rossini L, Meijer AH, Ouwerkerk PB (2014) Interaction between the GROWTH-REGULATING FACTOR and KNOTTED1-LIKE HOMEOBOX families of transcription factors. Plant Physiol **164:** 1952-1966

Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. Nature methods **9:** 357-359

Li Z, Baldwin CM, Hu Q, Liu H, Luo H (2010) Heterologous expression of Arabidopsis H -pyrophosphatase enhances salt tolerance in transgenic creeping bentgrass (*Agrostis stolonifera* L.). Plant, Cell Environ **33:** 272-289

Li W, Godzik A (2006) Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics **22:** 1658-1659

Liao Y, Smyth GK, Shi W (2014) featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics **30:** 923-930

Liu H, Guo S, Xu Y, Li C, Zhang Z, Zhang D, Xu S, Zhang C, Chong K (2014) OsmiR396d-regulated OsGRFs function in floral organogenesis in rice through binding to their targets OsJMJ706 and OsCR4. Plant Physiol **165:** 160-174

Long JA, Moan EI, Medford JI, Barton MK (1996) A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of Arabidopsis. Nature **379:** 66-69

Luo H, Hu Q, Nelson K, Longo C, Kausch AP (2004a) Controlling transgene escape in genetically modified grasses. Molecular Breeding of Forage and Turf. Springer, pp 245-254

Luo H, Hu Q, Nelson K, Longo C, Kausch AP, Chandlee JM, Wipff JK, Fricker CR (2004b) Agrobacterium tumefaciens-mediated creeping bentgrass (Agrostis stolonifera L.) transformation using phosphinothricin selection results in a high frequency of single-copy transgene integration. Plant Cell Rep **22:** 645-652

Ma C, Burd S, Lers A (2015) miR408 is involved in abiotic stress responses in Arabidopsis. Plant J **84:** 169-187

Omidbakhshfard MA, Proost S, Fujikura U, Mueller-Roeber B (2015) Growth-Regulating Factors (GRFs): A Small Transcription Factor Family with Important Functions in Plant Biology. Mol Plant **8**:998-1010

Osnato M, Stile MR, Wang Y, Meynard D, Curiale S, Guiderdoni E, Liu Y, Horner DS, Ouwerkerk PB, Pozzi C, Muller KJ, Salamini F, Rossini L (2010) Cross talk between the KNOX and ethylene pathways is mediated by intron-binding transcription factors in barley. Plant Physiol **154:** 1616-1632

Qin Z, Chen J, Jin L, Duns GJ, Ouyang P (2015) Differential Expression of miRNAs Under Salt Stress in *Spartina alterniflora* Leaf Tissues. Journal of Nanoscience and Nanotechnology **15:** 1554-1561

Qiu QS, Guo Y, Dietrich MA, Schumaker KS, Zhu JK (2002) Regulation of SOS1, a plasma membrane Na+/H+ exchanger in *Arabidopsis thaliana*, by SOS2 and SOS3. Proc Natl Acad Sci USA **99:** 8436-8441

Quan R, Lin H, Mendoza I, Zhang Y, Cao W, Yang Y, Shang M, Chen S, Pardo JM, Guo Y (2007) SCABP8/CBL10, a putative calcium sensor, interacts with the protein kinase SOS2 to protect Arabidopsis shoots from salt stress. Plant Cell **19:** 1415-1431

Quintero FJ, Martinez-Atienza J, Villalta I, Jiang X, Kim WY, Ali Z, Fujii H, Mendoza I, Yun DJ, Zhu JK, Pardo JM (2011) Activation of the plasma membrane Na^+/H^+ antiporter Salt-Overly-Sensitive 1

(SOS1) by phosphorylation of an auto-inhibitory C-terminal domain. Proc Natl Acad Sci USA **108:** 2611-2616

Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics **26:** 139-140

Rodriguez RE, Mecchia MA, Debernardi JM, Schommer C, Weigel D, Palatnik JF (2010) Control of cell proliferation in *Arabidopsis thaliana* by microRNA miR396. Development **137:** 103-112

Sharma P, Jha AB, Dubey RS, Pessarakli M (2012) Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. J Bot **2012**, Article 217037, DOI: 10.1155/2012/217037

Thomas J, Sepahi M, Arendall B, Bohnert H (1995) Enhancement of seed germination in high salinity by engineering mannitol expression in *Arabidopsis thaliana*. Plant, Cell Environ **18:** 801-806

Trapnell C, Pachter L, Salzberg SL (2009) TopHat: discovering splice junctions with RNA-Seq. Bioinformatics **25:** 1105-1111

Uozumi N, Schroeder JI (2010) Ion channels and plant stress: past, present, and future. Ion Channels and Plant Stress Responses. Springer, pp 1-22

Vollbrecht E, Reiser L, Hake S (2000) Shoot meristem size is dependent on inbred background and presence of the maize homeobox gene, knotted1. Development **127:** 3161-3172

Wang X, Zeng J, Li Y, Rong X, Sun J, Sun T, Li M, Wang L, Feng Y, Chai R (2015) Expression of TaWRKY44, a wheat WRKY gene, in transgenic tobacco confers multiple abiotic stress tolerances. Front Plant Sci **6:** 615

Wu SJ, Ding L, Zhu JK (1996) SOS1, a Genetic Locus Essential for Salt Tolerance and Potassium Acquisition. Plant Cell **8:** 617-627

Yang F, Liang G, Liu D, Yu D (2009a) Arabidopsis miR396 mediates the development of leaves and flowers in transgenic tobacco. J Plant Biol **52:** 475-481

Yang Q, Chen Z, Zhou X, Yin H, Li X, Xin X, Hong X, Zhu J, Gong Z (2009b) Overexpression of SOS (Salt Overly Sensitive) genes increases salt tolerance in transgenic Arabidopsis. Mol Plant **2:** 22-31

Yang CY, Huang YH, Lin CP, Lin YY, Hsu HC, Wang CN, Liu LY, Shen BN, Lin SS (2015) MicroRNA396-Targeted SHORT VEGETATIVE PHASE is required to repress flowering and is related to the development of abnormal flower symptoms by the Phyllody Symptoms1 Effector. Plant Physiol **168:** 1702-1716

Young MD, Wakefield MJ, Smyth GK, Oshlack A (2010) Method Gene ontology analysis for RNA-seq: accounting for selection bias. Genome Biol **11:** R14

Yuan S, Li Z, Li D, Yuan N, Hu Q, Luo H (2015) Constitutive Expression of Rice MicroRNA528 Alters Plant Development and Enhances Tolerance to Salinity Stress and Nitrogen Starvation in Creeping Bentgrass. Plant Physiol **169:** 576-593

Yue Y, Zhang M, Zhang J, Duan L, Li Z (2012) SOS1 gene overexpression increased salt tolerance in transgenic tobacco by maintaining a higher K^+/Na^+ ratio. J Plant Physiol **169:** 255-261

Zhang HX, Hodson JN, Williams JP, Blumwald E (2001) Engineering salt-tolerant Brassica plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. Proc Natl Acad Sci USA **98:** 12832-12836

Zhao T, Zhang J, Liang L, Ma Q, Chen X, Zong J, Wang G (2015) Expression and Functional Analysis of WRKY Transcription Factors in Chinese Wild Hazel, *Corylus heterophylla* Fisch. PloS ONE **10:** e0135315

Zhou J, Liu M, Jiang J, Qiao G, Lin S, Li H, Xie L, Zhuo R (2012) Expression profile of miRNAs in *Populus cathayana* L. and Salix matsudana Koidz under salt stress. Mol Biol Rep **39:** 8645-8654

CHAPTER IV - MIR396 IS INVOLVED IN FLOWER DEVELOPMENT AND PLANT RESPONSE TO VERNALIZATION

Abstract

MicroRNA396 (MiR396) has been suggested to regulate flower development by targeting *growth-regulating factor*s (*GRFs*) in annual species. To study the impact of miR396 on flower development in perennial species, we generated a rice miR396c overexpression construct and introduced it into creeping bentgrass (*Agrostis stolonifera* L.). Overexpression of miR396 leads to male sterility in transgenic (TG) creeping bentgrass plants, including short filaments, indehiscent anthers, and immature pollen grains. Most significantly, TG plants bypass the vernalization requirement for flowering, indicating that miR396 is involved in flowering time control in response to vernalization. Four genes from GRF family identified as potential targets of miR396 in creeping bentgrass are down-regulated in TG plants. The expression patterns of the closest orthologs of *VRN1*, *VRN2* and *VRN3*/*FT* are characterized in WT and transgenic plants during short-day (SD), long-day (LD), and cold exposure. The three genes in WT plants show similar expression patterns to those observed in wheat and barley during SD to LD, and SD to cold conditions. Compared to WT controls, miR396 TG plants show induced expression in *VRN1* and *VRN3*, but repressed expression in *VRN2* under SD to LD conditions without vernalization, which is associated with modified expression of methyltransferase genes. Additionally, a global view of the impacts on miR396 in reproductive development and vernalization was elucidated through RNA-seq analysis.

Our results point to the great potential of controlling flowering time through manipulating miR396 to contribute to agriculture productivity.

4.1 Introduction

Flowering is a crucial phase to determine plant reproductive success. Many crop species require prolonged exposure to the winter cold or vernalization to promote their vegetative-to-reproductive stage transition. However, vernalization largely limits crop growth region as well as flowering time due to varied winter temperature from place to place and from year to year. Therefore, it is highly valuable to study the molecular mechanisms of vernalization and develop new strategies to control flowering time.

Currently, the vernalization requirement at molecular level has been well studied in *Arabidopsis*. *FLOWERING LOCUS C* (*FLC*), a MADS box gene serves as a repressor of flowering in Arabidopsis (Michaels and Amasino, 1999). It suppresses flowering promoters *FLOWERING LOCUS T* (*FT*), *FLOWERING LOCUS D* (*FD*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) in leaves and meristems, respectively (Searle, et al., 2006). The process of vernalization leads to *FLC* silencing through reduced histone acetylation and increased histone methylation (H3K9 and H3K27) (Bastow, et al., 2004b; Sung and Amasino, 2004). Under a long-day (LD) photoperiod, *FT* transcripts are increased and move from leaves to the apical meristems to interact with *FD* and thereby initiate flowering by activating *SOC1* (Amasino, 2010). Interestingly, genes involved in the vernalization requirement are different between Arabidopsis and the cereals wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare*

L.). Thus far, a molecular framework for flowering time control in response to vernalization has been established in wheat and barley through identification and characterization of *VERNALIZAITON1* (*VRN1*), *VRN2*, and *VRN3* (Distelfeld, et al., 2009a; Greenup, et al., 2009). *VRN1* is a MADS-box transcription factor gene, which regulates the vegetative to reproductive phase transition (Danyluk, et al., 2003). *VRN2*, a flowering repressor, encodes a CCT domain and zinc finger containing protein not related to FLC (Yan, et al., 2004). *VRN3*, the ortholog of *FT* in wheat and barley, has a similar mechanism in promoting flowering as *FT* does in Arabidopsis (Li and Dubcovsky, 2008). Before vernalization, high levels of *VRN2* repress the florigen *VRN3* to prevent flowering (Yan, et al., 2004). During cold exposure, *VRN1* is activated through chromatin modifications, including a decrease in H3K27 methylation and an increase in H3K4 methylation (Oliver, et al., 2009). High levels of *VRN1* down-regulate *VRN2* during and after vernalization in leaves and meristems, which facilitates the accumulation of *VRN3* in leaves by LD photoperiod after vernalization (Distelfeld, et al., 2009b; Chen and Dubcovsky, 2012). *VRN3* then moves to shoot apex to maintain the high levels of *VRN1* and induces flowering (Distelfeld, et al., 2009a). Recently, the molecular mechanisms of vernalization response have been gradually uncovered in *Brachypodium distachyon*, a small temperate grass in the subfamily Pooideae as wheat and barley. In Brachypodium, *VRN1* and *VRN3*/*FT* have similar mechanisms as those in wheat and barley, whereas *VRN2* is induced during prolonged cold (Ream, et al., 2014), indicating that although within the same subfamily, the molecular mechanism of plant vernalization response is not conserved.

Accumulating evidence has demonstrated that microRNAs (miRNAs) are involved in flower development, including floral organ identity and polarity, floral organ size and shape, inflorescence development, male and female fertility, and flowering time control (Nag and Jack, 2010; Bergonzi, et al., 2013; Zhou, et al., 2013; Luo, et al., 2013). During floral organ formation, miR172 is critical in regulating the inner floral whorl patterning and lodicules development through targeting *APETALA2* (*AP2*) genes in Arabidopsis and the cereals rice and barley, repectively (Luo, et al., 2013). Besides, miR172 is also involved in flowering time control. Overexpression of miR172 causes increased accumulation of *FT* and early flowering, while overexpression of any one of the four *AP2* transcription factor genes, *TOE1*, *TOE2*, *SNZ*, or *SMZ* leads to late flowering (Aukerman and Sakai, 2003; Jung, et al., 2007). Another study showed that overexpression of miR156 leads to a prolonged juvenile stage, and a delayed flowering stage, while constitutively expressing miR156 targets, *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) promotes the transition from juvenile to adult stage and leads to early flowering (Wu and Poethig, 2006; Wu, et al., 2009). Recently, an age-dependent flowering pathway, which regulates the abundance of miR156 and miR172, has been identified in *Cardamine flexuosa* (Zhou, et al., 2013). This polycarpic perennial species does not respond to winter cold until it is five-week-old. Levels of miR156 decrease dramatically with time, which allows the accumulation of its target *SPLs* with high abundance to activate miR172. Up-regulated miR172 represses *AP2* and promotes the expression of floral integrator *SOC1*, and thereby induces flowering. Though miRNAs are implicated in flowering time control through integrating age and vernalization cues,

there has been no evidence suggesting that they are directly involved in the vernalization pathway.

MiR396 is an evolutionarily conserved miRNA targeting transcription factors, *growth-regulating factor*s (*GRFs*), involved in plant development at both vegetative and reproductive stages (Liu, et al., 2009b; Omidbakhshfard, et al., 2015). In Arabidopsis, the GRF family comprises nine members, seven of which have miR396 target sites (Jones-Rhoades and Bartel, 2004). Morphologically, transgenic plants constitutively expressing miR396 displayed shorter stature and narrower leaves than wild type (WT) controls in Arabidopsis and tobacco because of reduced cell numbers in leaf tissue (Kim, et al., 2003; Yang, et al., 2009; Rodriguez, et al., 2010). The similar phenotype was also observed in *atgrf1 atgrf2 atgrf3* triple mutants (Kim, et al., 2003). Besides its impact on plant vegetative growth, miR396 also controls plant development in the reproductive stage. Overexpression of miR396 resulted in stigmatoid anthers or a fasciated style with multiple stigma structures in transgenic tobacco (Baucher, et al., 2013), pistil abnormalities in transgenic Arabidopsis (Liang, et al., 2014), or open husks and long lemmas in transgenic rice (Liu, et al., 2014). However, the impact of miR396 on floral organ development in perennial species remains elusive. In addition, there has been no report indicating the involvement of miR396 in flowering time control or vernalization pathway in any plant species.

To investigate the role of miR396 in flower development in perennial species, we generated transgenic creeping bentgrass (*Agrostis stolonifera* L.) overexpressing a rice

miR396 gene, *Os-miR396c*. Our data indicate that four *GRF* genes identified as potential targets of miR396 in creeping bentgrass show repressed expression in transgenic plants, and transgenic miR396 plants exhibit stamen defects, including short filaments, indehiscent anthers, and immature pollen grains. Interestingly, compared to WT controls, miR396 TG plants also bypass vernalization to flower under long day (LD) conditions. To study the molecular mechanism of miR396-mediated flowering time control, expression profiles of miR396 and its targets were analyzed under the conditions of exposing plants to short day (SD) followed by transferring them to LD (referred hereafter as SD-LD), and exposure of SD-grown plants to prolonged cold followed by LD (referred hereafter as SD-cold-LD). In addition, the expression patterns of the closest orthologs of *VRN1*, *VRN2* and *VRN3*/*FT* are characterized in WT and TG creeping bentgrass in SD-LD and SD-cold-LD conditions. In WT plants, *VRN1*, *VRN2*, and *VRN3* show similar expression patterns as in wheat and barley under SD-LD and SD-cold-LD conditions. Without vernalization, *VRN1* and *VRN3* show induced expression, whereas *VRN2* is repressed under SD-LD conditions in TG plants compared to WT controls. Furthermore, a global view of the impacts of miR396 on reproductive development and vernalization was elucidated through RNA-seq analysis. Our results point to the great potential of controlling flowering time through manipulating miR396 to contribute to agriculture productivity.

4.2 Results

Overexpression of miR396c eliminates vernalization, but not long-day requirements for flowering

Creeping bentgrass is a cool season turfgrass, which requires vernalization, a prolonged plant exposure to the winter cold for the competence to flower. A previous study on colonial bentgrass (*Agrostis capillaris*), another species within the same genus as creeping bentgrass, revealed that 15 weeks of cold treatment (SD, 3-12°C or LD, 3-6°C) is required for inducing flowering (Heide, 1994). To determine the length of cold treatment required to saturate the vernalization response in creeping bentgrass, two replicates of WT and *Os-miR396c* transgenic creeping bentgrass grown under SD conditions (14-h light, 25°C /10-h dark, 17°C) were subjected to cold exposure (8-h light/16-h dark, 5°C) for 0, 11, 12, 13, 14, 15, 16, 17, and 18 weeks followed by shifting plants to LD conditions (16-h light, 25°C /8-h dark, 17°C). The results showed that WT plants do not flower under LD conditions until 15-week or longer cold exposure, whereas TG plants flower under LD conditions with or without cold exposure (from 0 to 18 weeks), indicating that TG plants overexpressing *Os-miR396c* are able to flower, bypassing the vernalization requirement (Figure 4.1). However, both TG plants (with or without vernalization) and vernalized WT controls require 4 weeks of LD (16-h light/ 8-h dark) induction for inflorescence emergence (Figure 4.2a, d). The longer cold treatment of 18 weeks does not accelerate flowering in WT plants (data not shown).

We then investigated the effect of photoperiod on flowering time in both WT and miR396 TG plants and found that neither TG plants without vernalization nor vernalized WT plants can flower under SD condition (14 h light). However, inflorescence emergence can be observed 4 or 3 weeks after a photoperiod of 16 or 24h, indicating that overexpression of miR396 does not affect plant response to photoperiod. Both WT and

miR396 TG plants are sensitive to photoperiod, and longer photoperiod leads to more rapid flowering.

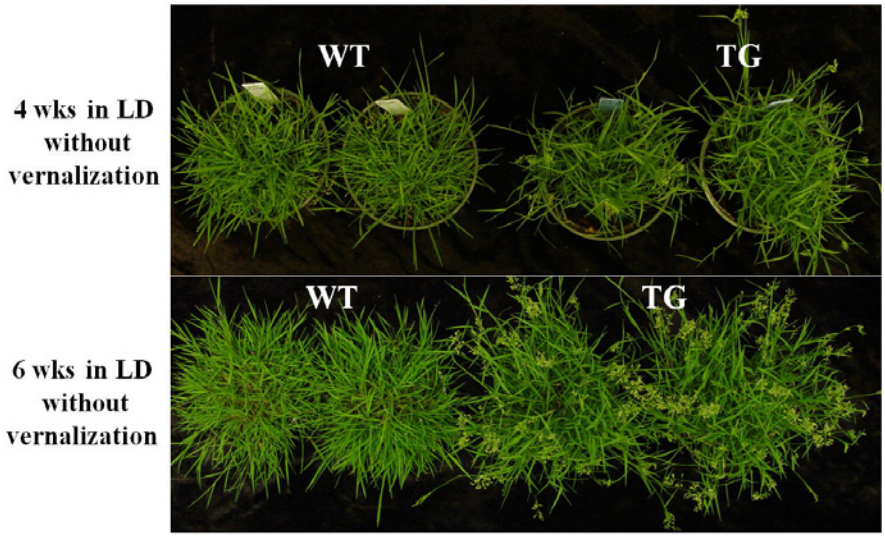

Figure 4.1 *Osa-miR396c* transgenic creeping bentgrass flowers without vernalization. Performance of WT and TG plants under 16-h photoperiod induction for four and six weeks without vernalization.

Overexpression of miR396c alters flower development that is associated with modified expression of target genes GRFs

To further investigate the impact of miR396 on creeping bentgrass reproductive growth, we compared floral organ development between WT and TG plants. WT and TG flowers were photographed 4, 6, and 8 weeks after LD following 15-week vernalization. During florescence emergence (4-week in LD) and anthesis (6-week in LD), the spikes of transgenic plants are curly in comparison with those of the WT controls (Figure 4.2a, b, d, e). Two weeks later, the panicles of the WT plants become reddish to purple, while those of the miR396 transgenics remain green (Figure 4.2c, f).

Microscopic analysis of the floralets in WT and TG plants during anthesis reveals that at the 6th and the 8th week in LD, miR396 TG plants exhibit defects in filament elongation and dehiscence, i.e., the filaments of the TG plants are shorter than those of the WT controls and the TG anthers do not normally dehisce (Figure 4.2g-j). Statistical analysis of the anther length indicates that TG anthers are significantly longer than those of the WT controls (Figure 4.2k, l).

Next, we examined pollen viability from both TG and WT control plants. Since pollen cannot be released by TG plants, we manually opened the fully developed WT and TG anthers before dehiscence and stained pollen with 2% (w/v) potassium iodide. As shown in Figure 4.2m and n, WT pollen is circular and darkly stained (Figure 4.2n), whereas TG pollen has varied shapes and is only lightly stained (Figure 4.2n), indicating that pollen of transgenic plants is sterile.

Accumulating evidence indicates that miR396 plays an important role in floral organ development by post-transcriptionally repressing the expression of *GRFs* (Liu, et al., 2014; Liang, et al., 2014). Seven of the nine *GRF* family members in Arabidopsis and ten of the twelve *GRFs* in rice have miR396 target sites (Debernardi, et al., 2012). In creeping bentgrass, we identified four *GRF* orthologs with miR396 binding sites. They are *AsGRF3*, *AsGRF4*, *AsGRF5*, and *AsGRF6* (Figure 3.8a). Their expressions are all repressed in miR396 TG plants compared with that in WT controls (Figure 3.8b), suggesting the participation of *GRFs* in modulating flower development in creeping bentgrass.

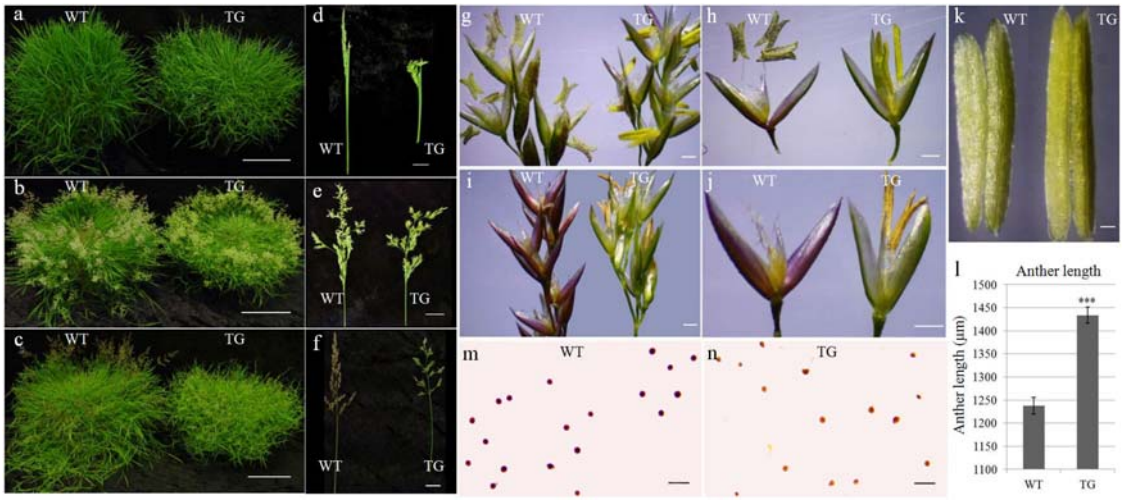

Figure 4.2 Flower development between WT and TG plants. Vernalized WT and TG plants under LD conditions for (a) four weeks, (b) six weeks, and (c) eight weeks. Scale bar, 10 cm. WT and TG spikelets after LD induction for (d) four weeks, (e) six weeks, and (f) eight weeks. Scale bar, 1 cm. Close up of WT and TG spikelets at (g) six weeks and (i) eight weeks after LD induction. Scale bar, 500 µm. Representative WT and TG floralets at (h) six weeks and (j) eight weeks after LD induction. Scale bar, 500 µm. (k) Representative anthers of WT and TG plants. Scale bar, 100 µm. (l) Anther lengths of WT & transgenic turfgrass overexpressing os-miR396c were measured at the same stage after flowering. Data are showed as means (n=6) with standard error. A significant difference between WT and TG anther length was indicated with asterisks at $P<0.001$ by Student's t-test. (m) WT and (n) TG pollen was stained with potassium iodide. Scale bar, 100 µm.

MiR396 is induced by LD photoperiod and vernalization

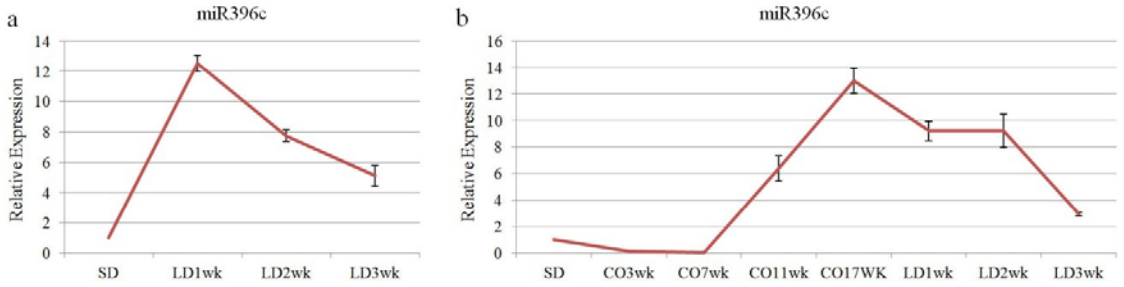

Figure 4.3 Expression profiles of miR396c under SD-LD, and SD-cold-LD conditions. (a) Stem-loop RT-qPCR analysis of miR396 expression in WT plants under SD conditions and in LD for 1-3 weeks without vernalization. (b) Stem-loop RT-qPCR analysis of miR396 expression in WT plants in SD, then 17 weeks of cold treatment, and under LD conditions for three weeks. The relative changes in gene expression were calculated based on the $2^{-\Delta\Delta CT}$ method. *AsUBQ* was used as an endogenous control. Data are presented as means of three technical replicates, and error bars represent ±SE.

To decipher the molecular mechanisms underlying the miR396-mediated plant response to vernalizaton, we first examined how miR396 is regulated by different photoperiods and temperatures. To this end, we analyzed the expression levels of miR396 in WT plants under SD-LD and SD-cold-LD conditions. When switching from SD (14-h photoperiod) to LD (16-h photoperiod), the abundance of miR396 was significantly elevated (12 fold) after one week in LD, and then gradually declined, but remained higher than that under SD conditions (Figure 4.3a). When switching from SD to cold conditions, levels of miR396 first slightly declined at 3rd and 7th week, and then significantly induced, attaining 12 fold more than in SD 17 weeks after cold treatment (Figure 4.3b). When shifting to LD conditions (25°C at daytime/17°C at night), levels of miR396 gradually declined but remained significantly higher than that under SD conditions (Figure 4.3b). These results indicate that miR396 responds to and is induced by LD light regime and low temperature, suggesting its possible involvement in regulating plant response to these environmental cues.

Identification of VRN1, VRN2, and VRN3 orthologs in creeping bentgrass

In winter cereals, *VRN1*, *VRN2*, and *VRN3* are key genes in the vernalization process for accelerating flowering (Distelfeld, et al., 2009a; Greenup, et al., 2009). A similar mechanism has also been demonstrated in Brachypodium (Ream, et al., 2014). This raises the question of whether or not in creeping bentgrass, miR396 affects plant vernalization response through indirectly regulating *VRN* gene expression. Currently, creeping bentgrass vernalization response at the molecular level remains largely unknown. We

decided to examine the role the *VRN* genes play in plant vernalization response in this cool-season perennial turfgrass. To this end, we cloned the creeping bentgrass full-length cDNAs of the winter cereals orthologs of the *VRN1*, *VRN2* and *VRN3*/*FT* by 5' and 3' RACE (Rapid Amplification of cDNA Ends).

AsVRN1 encodes a 262-aa protein, which shares a high identity with its orthologs from wheat (72%), barley (68%), Brachypodium (73%), rice (72%), and Arabidopsis (68%). A neighbor-joining phylogenetic analysis of full-length amino acid sequences from these species divides *VRN1* orthologs into a monocot clade and a dicot clade (Figure 4.4a). *AsVRN1* is placed in the monocot clade as shown in Figure 4.4a. *VRN1* from grass is related to *APETALA1* (*AP1*)/ *CAULIFLOWER* (*CAL*)/ *FRUITFUL* (*FUL*), a subgroup of MADS-box transcription factors controlling the initiation of the vegetative to reproductive transition (Yan, et al., 2003; Preston and Kellogg, 2008). Sequence alignment revealed a conserved type-II MADS domain and a K-box (Figure 4.4d), which are characteristic of *AP1*/*CAL*/*FUL* clade. The high sequence similarity of the conserved MADS domain and K-box between *AsVRN1* and each *VRN1* ortholog suggests that *AsVRN1* is the *VRN1* putative ortholog in creeping bentgrass (Figure 4.4d).

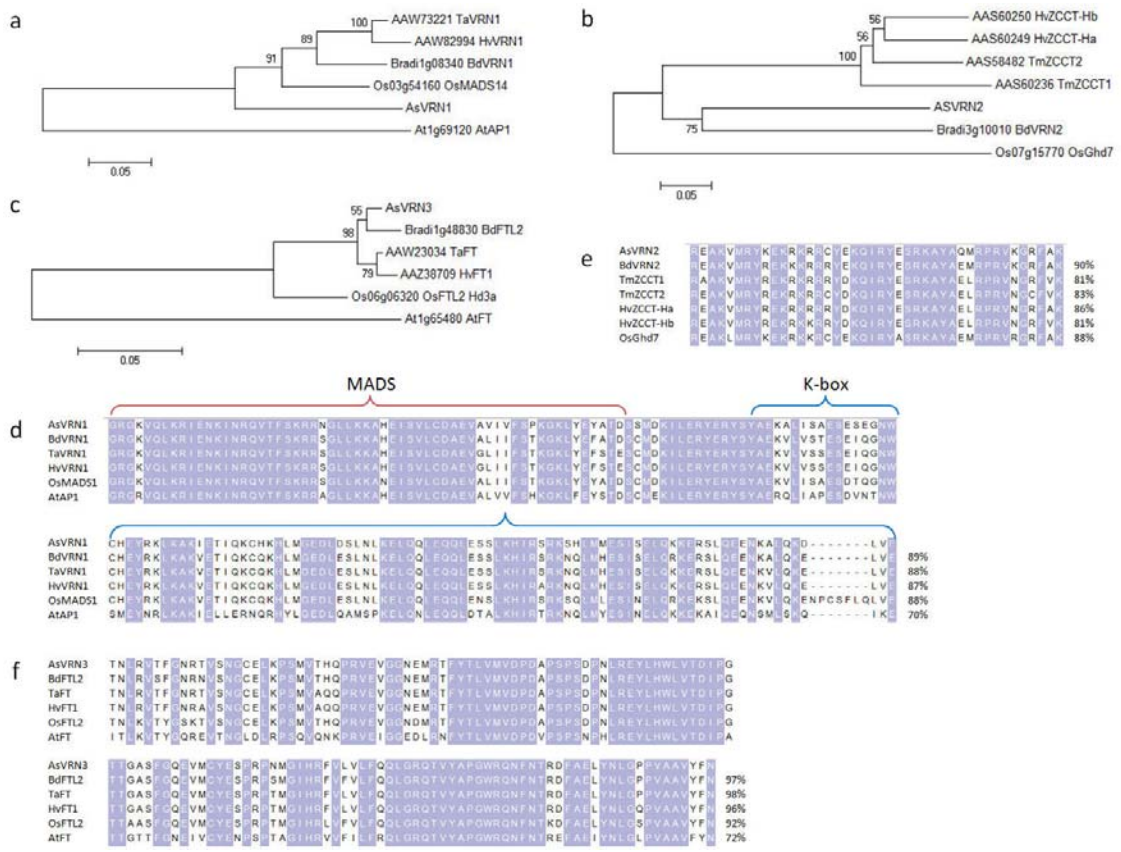

Figure 4.4 Phylogenetic analyses of VRN1, VRN2, and VRN3 proteins and sequence alignment of their conserved domains. Phylogenetic trees of (a) VRN1, (b) VRN2, and (c) VRN3 from creeping bentgrass, Brachypodium, wheat, barley, rice and Arabidopsis were built with the neighbor-joining method. Bootstrap values were derived from 1000 replications. Alignment of (d) type II subfamily of MADS domain and K-box, (e) CCT domain, and (f) PEBP (phorsphatidylethanolamine) domain among different plant species, including creeping bentgrass, Brachypodium, wheat, barley, rice and Arabidopsis. Species abbreviations: As=*Agrostis stolonifera*, Bd=*Brachypodium distachyon*, Ta=*Triticum aestivum*, Tm=*Triticum monococcum*, Hv=*Hordeum vulgare*, Os=*Oryza sativa*, and At=*Arabidopsis thaliana*. Similarities of conserved domains between creeping bentgrass and each of the orthologs are listed at the end of the alignment.

The flowering repressor *VRN2* in cereals behaves similarly to the MADS-box gene *FLC* in Arabidopsis, while they are unrelated. *VRN2* orthologs from wheat (*TmZCCT1* and *TmZCCT2*), barley (*HvZCCT-Ha* and *HvZCCT-Hb*), Brachypodium (*BdVRN2*), and rice (*OsGhd7*) are members of a *CONSTANS-like* superfamily containing a conserved CCT domain. Currently, the ortholog of *VRN2* in Arabidopsis is unknown. In creeping

bentgrass, we successfully cloned the full length of *AsVRN2*, which encodes a 213 aa protein. Amino acid sequences alignment between *AsVRN2* and other *VRN2* orthologs indicates that *AsVRN2* carries a CCT domain (Figure 4.4e). Phylogenetic analysis of the complete protein sequences of *VRN2* orthologs placed *AsVRN2* and *BdVRN2* into the same clade (Figure 4.4b), suggesting that *AsVRN2* is the *VRN2* putative ortholog.

In addition to the vernalization pathway, photoperiod, autonomy, hormones, and age pathways also participate in the plant vegetative to reproductive transition. These endogenous and environmental signals are sensed by plants and ultimately stimulate a set of flowering integrators. *VRN3*/*FT* is one of the flowering integrators, which belongs to phosphatidylethanolamine-binding protein (PEBP) gene family. *VRN3* cloned in creeping bentgrass encodes a 177-aa protein. Amino acid sequences alignment among *VRN3*/*FT* orthologs shows that *AsVRN3* carries a conserved PEBP domain with a length of 139-aa (Figure 4.4f). A phylogenetic tree was built with the complete amino acid sequence of *VRN3*/*FT* from creeping bentgrass, wheat, barley, Brachypodium, rice, and Arabidopsis. As Figure 4.4c shows, *VRN3*/*FT* from the grass subfamily Pooideae are grouped into one clade, while Brachypodium ortholog (*BdFTL2*) and *AsVRN3* reside in the same subclade, suggesting that *AsVRN3* is in fact a *VRN3*/*FT* ortholog in creeping bentgrass.

Effects of vernalization and different photoperiod regimes on VRNs in WT and TG creeping bentgrass

By cloning *VRNs* in creeping bentgrass, we seek to answer several questions. How are *VRNs* regulated in creeping bentgrass when exposed to prolonged cold and different

light regimes? Do *VRNs* have similar responses as in wheat, barley, or Brachypodium? What is the impact of miR396 on *VRNs*? What causes the elimination of vernalization requirement in TG plants? To answer these questions, we compared the expression profiles of *VRN1*, *VRN2*, and *VRN3* in SD-LD and SD-cold-LD conditions in WT and TG creeping bentgrass.

Without vernalization, *AsVRN1* expression in WT plants is low under SD, but significantly induced when shifted to LD during the first two weeks. Its expression declines at LD 3-week, but remains elevated compared to that under SD (Figure 4.5a, c), which is in consistent with the expression profile of *VRN1* in wheat (Dubcovsky, et al., 2006). In miR396 transgenic creeping bentgrass, *AsVRN1* is induced in LD and remains elevated at LD 3-week (Figure 4.5a, c). Interestingly, levels of *AsVRN1* are higher in TG plants vs. WT controls under both SD and LD conditions (Figure 4.5a, c), implying that *VRN1* is affected by miR396. During prolonged cold treatment, levels of *AsVRN1* are gradually increased and remain elevated following cold treatment in LD (Figure 4.5b, d), which is in agreement with previous studies in cereals wheat, barley, and Brachypodium (Sasani, et al., 2009; Ream, et al., 2014). *AsVRN1* in TG plants has a similar expression profile to that in WT controls, though its levels are higher under SD and LD than that in WT plants, but not at the saturated cold (cold 17-week; Figure 4.5b, d).

In barley, levels of *HvVRN2* (*HvZCCT-Ha* and *HvZCCT-Hb*) expression are higher when plants are grown in LD than in SD (Trevaskis, et al., 2006). Similarly in creeping bentgrass, levels of *AsVRN2* expression in WT plants increase significantly during the

first two weeks of LD induction, and then decline at LD 3-week (Figure 4.5a, c). In contrast, levels of *AsVRN2* in transgenic plants only slightly increase when switched from SD to LD (Figure 4.5a, c), and compared with WT controls, *AsVRN2* in TG plants is repressed under both SD and LD conditions (Figure 4.5a, c, suggesting that levels of *AsVRN2* expression are affected by miR396. It has previously been reported that during vernalization, *VRN2* transcript levels in both wheat and barley leaves decrease (Trevaskis, et al., 2006; Yan, et al., 2004). Consistent with this, *AsVRN2* expression in both WT and TG plants also gradually declines during vernalization, and then is elevated under LD conditions (Figure 4.5b, d).

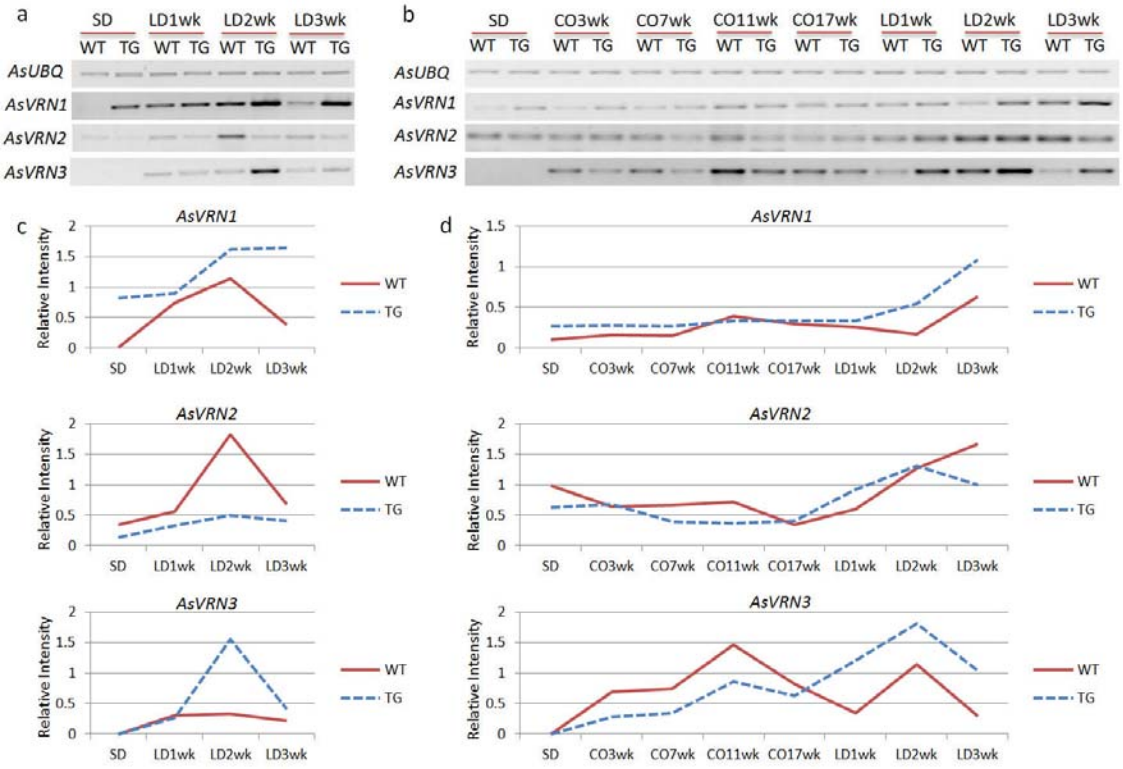

Figure 4.5 Expression profiles of *AsVRN1*, *AsVRN2*, and *AsVRN3* in SD-LD and SD-cold-LD conditions. (a) Semi-quantitative RT-PCR analysis of *AsVRN1, AsVRN2, and AsVRN3* gene expression in WT and TG plants under SD-LD conditions without vernalization. (b) Semi-quantitative RT-PCR analysis of *AsVRN1,*

AsVRN2, and AsVRN3 gene expression in WT and TG plants under SD-cold-LD conditions. *AsUBQ* was used as an endogenous control. Analysis of band intensity on electrophoresis gel is presented as relative ratios of *AsVRN1, AsVRN2, and AsVRN3* to *AsUBQ* under (c) SD-LD conditions and (d) SD-cold-LD conditions. The band intensity was quantified using ImageJ (Abràmoff, et al., 2004).

In wheat and barley, *VRN3* transcription is very low in SD and its expression is induced when plants are grown in LD (Turner, et al., 2005; Yan, et al., 2006). In WT creeping bentgrass, *VRN3* exhibits a similar expression pattern as in wheat and barley, i.e., *VRN3* is slightly induced when shifted from SD to LD (Figure 4.5a, c). In contrast, TG plants overexpressing miR396 exhibit significantly enhanced *VRN3* expression at LD 2-week, and their *VRN3* expression then declines at LD 3-week (Figure 4.5a, c). The dramatic upregulation of the flowering-promoting factor, *VRN3* suggests the transition of miR396 TG plants from vegetative to reproductive growth at LD 2-week. Besides the impact of day length on *VRN3* expression, vernalization has also been reported to upregulate *VRN3* in barley, wheat, and Brachypodium (Yan, et al., 2006; Ream, et al., 2014). In accordance with this, *AsVRN3* mRNA levels are elevated during prolonged cold and the following LD treatments in both WT and TG plants, indicating that *AsVRN3* plays a role analogous to *VRN3*/*FT* in wheat, barley, and Brachypodium.

Impacts of miR396 on histone methylation

Differential regulations of *VRNs* by photoperiod between WT and miR396 TG plants without vernalization indicate that miR396 plays a role in the vernalization pathway. Since VRN1 is an upstream regulator which represses the levels of *VRN2* to promote the expression of *VRN3* during and following vernalization, we speculate that *VRN1* is directly or indirectly regulated by miR396. The upregulation of *HvVRN1* during

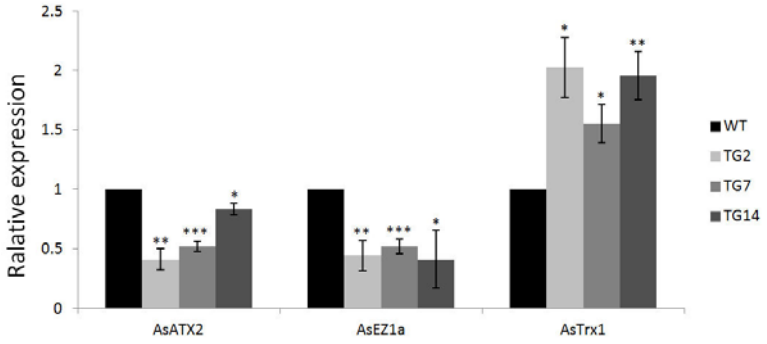

Figure 4.6 Expression levels of methyltransferases *AsATX2*, *AsEZ1a*, and *AsTrx1* in WT and TG plants under SD conditions. Real-time RT-PCR analysis of *AsATX2*, *AsEZ1a*, and *AsTrx1* expression in WT and three transgenic plants under SD conditions. *AsUBQ* was used as an endogenous control. Data are presented as means of three technical replicates, and error bars represent ±SE.

vernalization has been reported to be associated with an increase of active histone marks H3K4 trimethylation (H3K4me3) and a decrease of silent marks H3K27me3 at *HvVRN1* chromatin, while levels of *VRN2* and *VRN3* in barley are not altered by histone modifications (Oliver, et al., 2009). In winter wheat, *TaVRN1* is up-regulated during vernalization through an increased level of H3K4me3 but no change in the level of H3K27me3 (Diallo, et al., 2012). Therefore, it is hypothesized that *AsVRN1* might also be regulated by histone modifications during vernalization. In this study, higher levels of *AsVRN1* have been observed in TG plants than in WT controls under SD and LD without vernalization, which prompts us to examine if the levels of active and silent histone marks are different in WT and TG plants. Transcript levels of methyltransferases are compared between WT and three transgenic lines through quantitative RT-PCR analysis. *AsATX2* and *AsTrx1*, which mediate methylation at H3K4, are down- and up-regulated in three transgenic lines, respectively. *AsEZ1a*, which mediates methylation at H3K27, is

repressed in TG plants. The results imply that miR396 might be involved in histone methylation.

Effects of day length and vernalization on GRFs in WT and TG plants

In addition to the key genes in the vernalization pathway, transcript levels of miR396 putative targets were also analyzed under SD-LD and SD-cold-LD conditions in both WT and TG plants. MiR396 is significantly induced and remains elevated when transferring plants from SD to LD (Figure 4.3a). In contrast, levels of its targets, *AsGRF3* and *AsGRF4* are significantly down-regulated when switching from SD to LD conditions in both WT and TG creeping bentgrass (Figure 4.7a, c). Interestingly, expression profiles of *AsGRF5* and *AsGRF6* in WT plants do not show negative correlation with the levels of miR396 in SD-LD, suggesting that *AsGRF5* and *AsGRF6* might also be regulated by light-related factors besides their direct repressor miR396. In addition, levels of *AsGRF5* are higher in transgenics than in WT controls under LD induction (Figure 4.7a, c), suggesting that miR396 might impact *AsGRF5* by regulating other unknown factors. When exposing WT and TG plants to SD-cold-LD conditions, the expression the *AsGRF3* and *AsGRF4* show negative correlation with that of the miR396, whereas this negative correlation is not observed between the expression profiles of *AsGRF5*, *AsGRF6* and miR396, presumably due to the greater impacts of other factors on the expression of the *AsGRF5*, *AsGRF6* during cold and LD conditions.

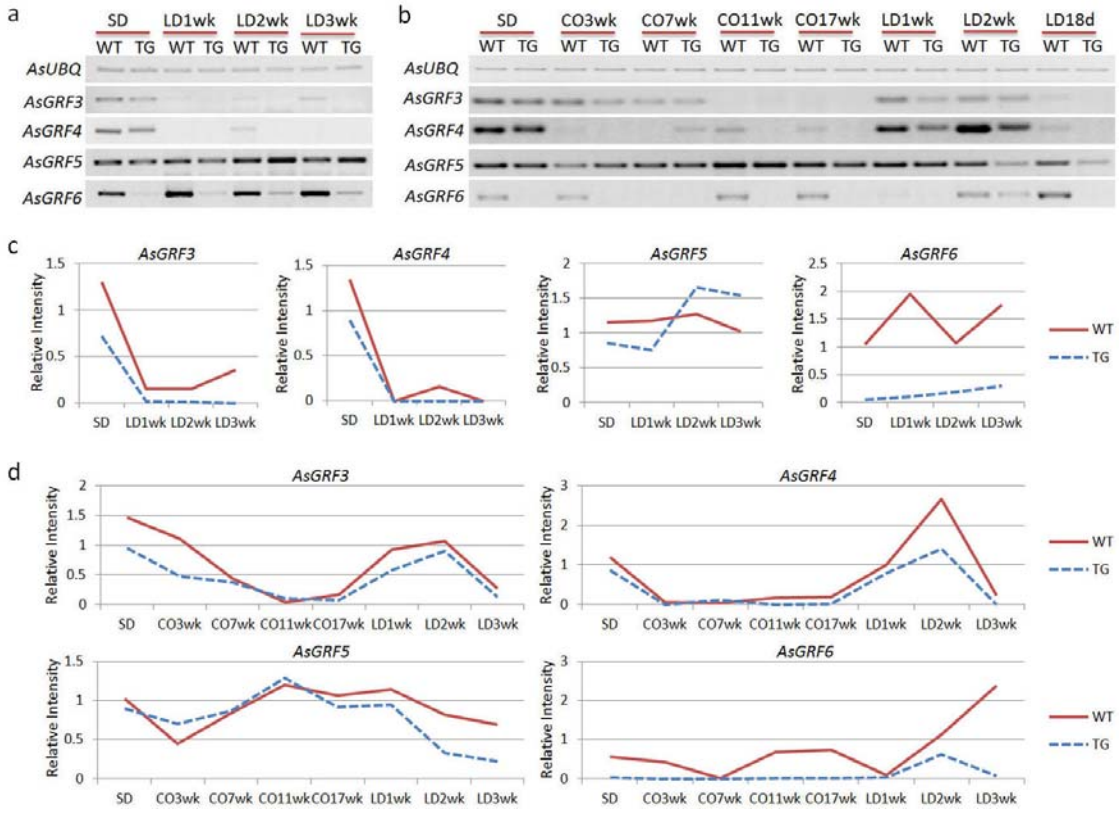

Figure 4.7 Expression profiles of the putative miR396c targets *AsGRF3, AsGRF4, AsGRF5,* and *AsGRF6 in* SD-LD and SD-cold-LD conditions. (a) Semi-quantitative RT-PCR analysis of *AsGRF3, AsGRF4, AsGRF5, and AsGRF6* gene expression profiles in WT and TG plants under SD-LD and (b) SD-cold-LD conditions. (c) Analyses of band intensity on electrophoresis gel are presented as relative ratio of *AsGRF3, AsGRF4, AsGRF5, and AsGRF6* to *AsUBQ* under SD-LD conditions and (d) SD-cold-LD conditions. The band intensity was quantified using ImageJ (Abràmoff, et al., 2004).

Genome-wide gene expression analysis in miR396 TG plants

To further understand how miR396 is involved in floral organ development and flowering time control, we performed RNA-seq analysis to study the differences in gene expression at genome level between WT controls and TG plants. Leaf and shoot apical meristem (SAM) samples were collected in LD 3-week (without vernalization) for cDNA library preparation and Illumina sequencing as described in Chapter III.

Gene ontology enrichment analysis

To identify the major functional categories which are represented in transgenics vs. WT controls, we performed gene ontology (GO) enrichment analysis. Figure 4.8 shows that 21 GO terms were significantly enriched (over-represented p-value < 0.05) in up-regulated ($\log_2$ FC >2) and down-regulated ($\log_2$ FC < -2) genes, respectively. Among others, the GO terms 'electron transport chain', 'carbohydrate metabolism', 'chloroplast thylakoid membrane', and 'ATP binding' are enriched in the up-regulated genes. Under LD 3-week induction without vernalization, TG plants are in the flower development stage while WT plants are still in vegetative growth. These enriched GO terms are related to energy generation and metabolism, which are fundamental for energy supply, carbon storage, and cell wall formation during flower development. This result is in close agreement with a study in other plant species during flower development (Singh, et al., 2013). The enriched GO terms in the down-regulated genes include 'cell division', 'DNA replication', 'regulation of transcription', 'nucleus', and 'DNA binding', which indicates that the processes of DNA replication and cell division are strongly repressed in TG plants overexpressing miR396. This result is consistent with the miR396-GRF system, which shows decreased cell number in miR396 transgenic leaves through repressing levels of *GRFs* (Liu, et al., 2009b).

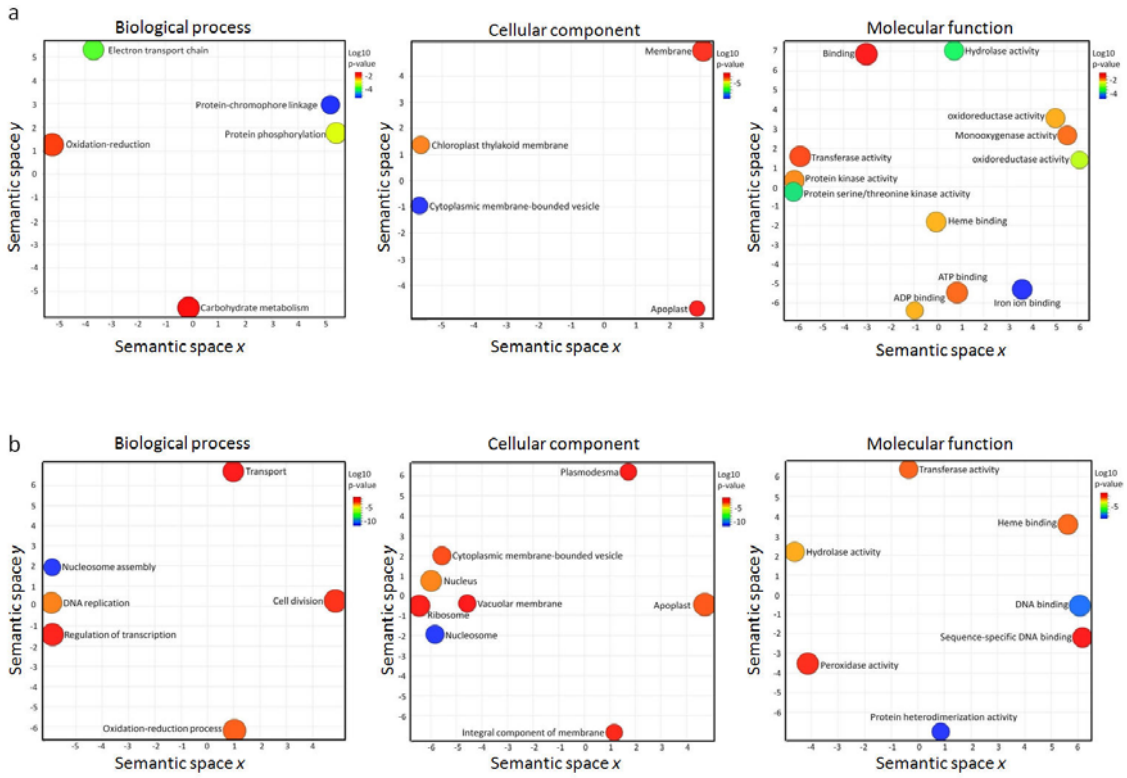

Figure 4.8 GO enrichment analysis. Significantly enriched GO terms for genes (a) up-regulated ($\log_2$ FC > 2) and (b) down-regulated ($\log_2$ FC < -2) in TG vs. WT are projected onto a two-dimensional semantic space with three GO categories, including biological process, cellular component, and molecular function. Each bubble represents a GO term. The closer the bubbles rest, the more related the GO terms are. The bubble color represents the significance of the enrichment. Bubbles with more general GO terms are larger.

Differential expression of transcription factor genes

MiRNAs are involved in various plant physiological processes through regulating their targets, most of which are transcription factors (TFs). In addition, many TFs are key regulators implicated in flowering time control and flower development. Thus, we performed differential expression analysis of genes encoding for TFs. The heatmap in Figure 4.9 shows 77 genes from 9 TF families are differentially expressed ($\log_2$ FC >1 or <-1, FDR corrected *p*-value < 0.05) in TG vs. WT plants, including NAC, MYB, MADS, GATA, E2F, bHLH, AP2, homeobox, and bZIP. Among them, the MADS-box TF

family, which consists of 10 up-regulated and 8 down-regulated members, is most represented.

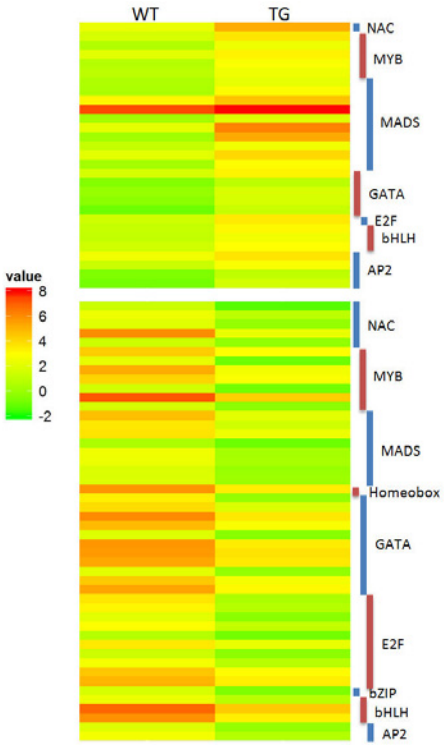

Figure 4.9 Differential expression of transcription factor genes at 3-week LD induction. Various transcription factor families with differential expression ($\log_2$ FC > 1 and $\log_2$ FC < -1 in upper and lower panel, respectively) are shown in the heatmap. The color scale represents the $\log_2$-transformation of the read count value.

The second most highly represented is GATA family (5 up-regulated and 11 down-regulated) followed by MYB TF genes (5 up-regulated and 7 down-regulated). MADS-box TFs play an essential role during plant flowering, which includes transition to flowering, petal and stamen specification, carpel and ovule development, pollen maturation and tube growth, and sepal and petal longevity (Smaczniak, et al., 2012). Many GATA family members play a predominant role in floral development. For example, *GNC* and *GNL* from GATAs are flowering repressors through regulating the

expression of florigen *SOC1*, while *HAN* serves as a floral morphology regulator through controlling the homeobox TF *WUSCHEL* (Behringer and Schwechheimer, 2015). MYB TFs are critical for floral asymmetry (Corley, et al., 2005). Other TF families, which exhibit significant differential expression, are also largely implicated in floral development (Ning, et al., 2015; Niu, et al., 2015; Zhao, et al., 2012; Ito, et al., 2012; Lee, et al., 2014; Abe, et al., 2005).

Differential expression of flower development and chromatin modification genes

Besides identifying the major functional categories overrepresented in transgenics, we are also interested in the flower development and chromatin modification-related genes in TG versus WT plants. The later may play a role in epigenetically regulating *VRN* gene expression to control flowering. Therefore, we determined the significantly enriched GO terms (over-represented p-value < 0.05), which relate to flower development and chromatin modifications. The corresponding differentially expressed genes (DEGs, $\log_2$ FC > 1 or $\log_2$ FC < -1, FDR corrected p-value < 0.05) from each GO term were selected to generate a heatmap (Figure 4.10). As shown in Figure 4.10, 84 genes were categorized into the flower development group, which includes processes of SAM specification, vegetative to reproductive transition, flower organ formation, anther dehiscence, and starch biosynthesis and metabolism; 69 genes are categorized into the chromatin modification group, including the processes of histone acetylation and methylation. These results provide evidence at the molecular level about the differences in reproductive transition and development we observed between TG plants and WT controls, such as

altered flowering time, anther dehiscence defect, pollen sterility, and different levels of *VRN1*.

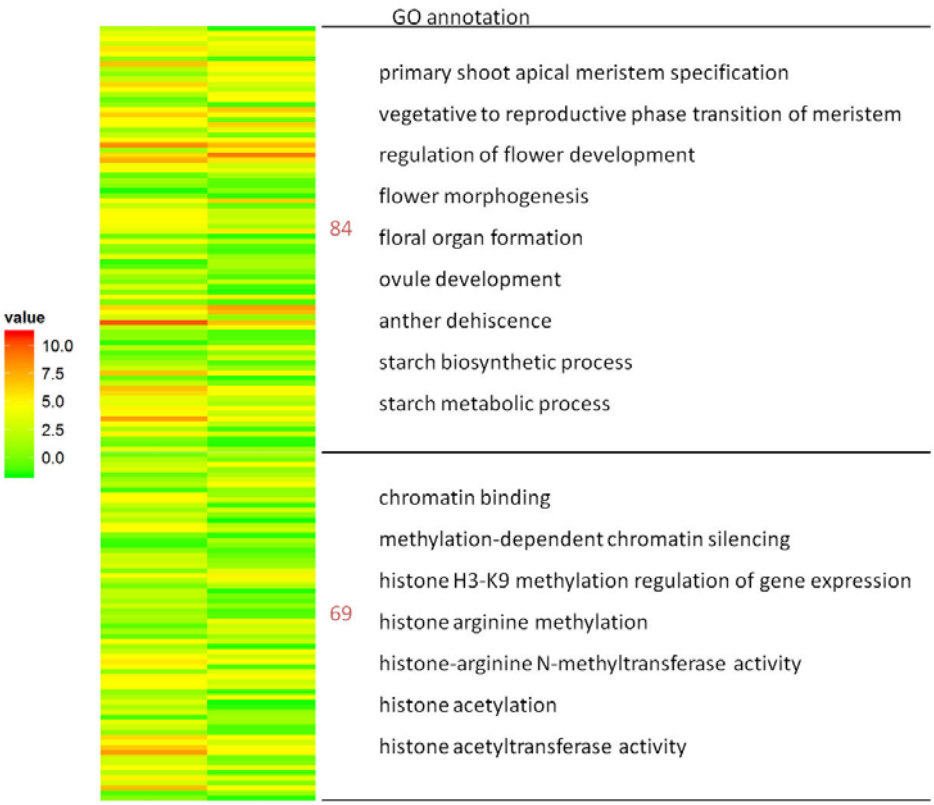

Figure 4.10 Differential expression of flower development and chromatin modification related genes. Significantly enriched GO terms ($\log_2$ FC > 1 or $\log_2$ FC < -1, FDR < 0.05), which relate to flower development and chromatin modification, in TG vs. WT data sets are listed. The corresponding differentially expressed genes (DEGs) of each enriched GO term were selected to generate the heatmap. The color gradient shows the $\log_2$-transformation of the read count value.

4.3 Discussion

MiR396-mediated flower development

Various studies have demonstrated that miR396 regulates plant leaf growth and floral organ development in both monocot and dicot species through targeting *GRFs*. High levels of miR396 or *grf* loss-of-function mutants always lead to the similar leaf

phenotype, which is smaller leaf areas due to reduced cell size or cell number (Kim, et al., 2003; Yang, et al., 2009; Rodriguez, et al., 2010). In contrast, overexpression of miR396 causes distinct floral organ defects in different plant species, such as open husks and long sterile lemmas, abnormal pistils, altered anther and carpel morphology in transgenic rice, Arabidopsis, and tobacco (Liu, et al., 2014). In this study, transgenic creeping bentgrass plants displayed abnormal stamen development, which includes reduced filament extension, dehiscence defects, and pollen sterility. The GRF family, a small transcription factor family, forms a complex with the transcriptional co-activators GRF-interacting factors (GIFs) to regulate downstream functional genes in leaf and SAM. It is likely that *GRF* members from different plant species target different genes in SAMs to regulate floral organ development, thereby leading to distinct flower phenotypes. In transgenic rice overexpressing *OsmiR396d*, for example, altered floral organ morphology was shown to result from the repressed expression of *OsGRF6* and *OsGRF10*, which targets a H3K9 demethylase gene *OsJMJ706* and a kinase gene *OsCR4* involved in the maintenance of the palea and lemma interlocking (Liu, et al., 2014). However, the regulatory networks between other GRF homologs and downstream targets in rice and other plant species remain unknown. Further identification of GRFs' targets in different plant species would provide information to better understand miR396-GRF module-mediated floral organ development.

Plant male sterility provides benefits not only in preventing pollen-mediated gene flow in transgenic plants, but also in blocking self-fertilization facilitating hybrid production for new breeding opportunities. Overexpression of *Os-miR396c* in creeping

bentgrass resulted in male sterility with plant defects of short filaments, indehiscent anthers, and immature pollen grains. Interestingly, the similar male sterile phenotype was also observed in transgenic Arabidopsis with repressed expression of a bHLH TF gene *MYC5* through overexpression of a MYC5-SRDX chimeric repressor, and in transgenic tobacco with ectopic expression of a soybean MADS-box TF gene, *GmMADS28* (Huang, et al., 2014; Figueroa and Browse, 2015). *MYB26* is responsible for anther dehiscence through regulating *NST1* and *NST2* from NAC TF family (Mitsuda, et al., 2005; Yang, et al., 2007). The TF families mentioned above are differentially expressed in miR396c TG plants and WT controls, implying that miR396 functions in stamen development through regulating other genes, especially TF genes. It is also plausible that the male sterile phenotype may be directly attributed to miR396-GRF module.

In the present study, the lemmas of WT spikelets turn reddish-brown when floral organs begin to senesce, whereas miR396 transgenic spikelets remain green (Figure 4.2i, j). The reddish-brown color in the spikelet results from the synthesis of anthocyanins and the degradation of chlorophyll during plant senescence. Given that oxidative stress triggers plant senescence, we speculate that overexpression of miR396 may have delayed flower senescence in transgenic plants by enhancing plant tolerance to oxidative stress. Our hypothesis is further supported by the result of GO enrichment analysis, in which the GO terms oxidation-reduction and oxidoreductase activity are enriched in up- and down-regulated genes (Figure 4.8). However, previous studies show that high levels of *GRF3* and *GRF5* delay leaf senescence in transgenic Arabidopsis (Debernardi, et al., 2014; Gonzalez, et al., 2010), implying that constitutive expression of miR396 might trigger,

other than delay, leaf senescence. The controversial results could be attributed to the species-specific function of miR396-GRF module. In addition to the direct regulation of plant senescence via the miR396-GRF pathway, differential regulation of anthocyanins accumulation in WT and TG plants is another possibility. TF families MYB and bHLH MYC are implicated in the process of anthocyanins synthesis (Stommel, et al., 2009). Since MYB and bHLH TF families show differential expression in transgenics and WT controls as shown in Figure 4.9, it is likely that the anthocyanins synthesis pathway is affected in transgenics overexpressing miR396, which leads to the green spikelets in TG plants.

Elimination of vernalization requirement

In temperate climate, flowering time must coincide with optimal environmental conditions for reproductive success. Many plants sense and respond to prolonged winter cold and flower next spring when day length increases. However, winter temperature varies from year to year and from place to place, which largely limit agricultural practices. Genetic modifications of plants to eliminate vernalizaiton requirement overcome these constraints and expand regions for agricultural practices. In addition, flexible flowering time is an advantage in reducing damages from certain environmental stresses. Furthermore, effective elimination of vernalization allows biennials and perennials to flower at the first year, which accelerate the introgression of new agronomic traits in breeding process.

Currently, the elimination of the vernalization requirement has been accomplished through genetic engineering of vernalization pathway genes in both dicot and monocot species. In Arabidopsis, *FRIGIDA* (*FRI)* complex positively regulates *FLC* to repress flowering (Choi, et al., 2011). Evidence shows that the vernalization requirement is eliminated in plants containing mutations in the components of the complex, such as *FRI*, *SUPPRESSOR OF FRI4*, *FRI ESSENTIAL1*, and *FLC EXPRESSOR* (Schmitz, et al., 2005; Kim and Michaels, 2006; Kim, et al., 2006). The similar result is also obtained in wheat with deletions or mutations in the monocot flowering repressor *VRN2* (Distelfeld, et al., 2009b). Besides the flowering repressor, modification of the flowering activator *VRN1* leads to the same phenotype. The wheat gene with a mutation in the *VRN1* promoter or large deletions in its first intron could bypass vernalization requirement (Zhang, et al., 2012; Chu, et al., 2011; Fu, et al., 2005). The same phenotype could also be achieved through activating flower integrator. In Medicago, vernalization and the following LD conditions induce *FT* ortholog *FT1a*. A gain-of-function mutant with high levels of *FT1a* transcripts exhibits up-regulated *SOC1* and *FULb*, resulting in early flowering without vernalization requirement in LD (Jaudal, et al., 2013). In this study, levels of the vernalization pathway genes were altered in miR396 TG creeping bentgrass, suggesting that the elimination of vernalization is accomplished through regulating *VRNs*. In SD-LD conditions, the flowering activator *VRN1* remained elevated, while the flowering repressor *VRN2* remained repressed in TG plants. Based on the vernalization pathway established in wheat and barley, we conclude that the repressed *VRN2* expression is caused by high levels of *VRN1*. In comparison with WT controls, TG plants

displayed significantly up-regulated *VRN3* expression in LD when switched from SD conditions. This might result from the repression of *VRN2* expression, thereby promoting flowering of TG plants bypassing vernalization requirement.

A MiRNA-mediated early flowering phenotype has been observed through regulating flowering integrators, such as *SOC1* and *FT*. For example, the Pooideae-specific miRNA, miR5200 targets *FT* mRNA in Brachypodium (Wu, et al., 2013). Artificial interruption of miR5200 in SD leads to early flowering (Wu, et al., 2013). In the biennial-to-perennial plant *C. flexuosa*, the miR156-*SPL*s-miR172-*AP2* module regulates *SOC1* to control flowering time (Zhou, et al., 2013). Wild-type *C. flexuosa* does not respond to vernalization until plants are 5-week-old. Artificial interruption of miR156 or overexpression of miR172 results in an early response to cold or even the elimination of the vernalization requirement. In this study, we speculate that miR396 may be critical for the elimination of vernalization through regulating vernalization pathway in creeping bentgrass, although the mechanisms behind this remain unknown. Stem-loop RT-qPCR analysis shows that miR396 in WT plants was induced in LD and prolonged cold conditions. Thus, it is hypothesized that prolonged cold leads to the up-regulation of miR396, which then turns unvernalized plants to the vernalized state through modulating its direct target(s) or other unknown regulatory element(s), and then LD induces floral homeotic genes in the "ABC" model to specify floral organs. High levels of miR396 in TG plants result in the "vernalized state" without cold treatment, thereby flowering after LD induction. Since miR396 is a conserved miRNA existing in both monocot and dicot species, we also investigated whether miR396-mediated elimination of vernalization

requirement can be applied to dicot perennial species. Transgenic *C. flexuosa* overexpressing miR396 did not flower without vernalization treatment (Figure A-8). The result indicates that the conserved miRNA also has species-specific functions. Given the fact that monocot and dicot species establish their vernalization requirement by recruiting different genes, it is not surprising that miR396 is not involved in vernalization pathways of this dicot species. Further analysis in perennial monocot species would reveal whether the role of miR396 in flowering time control is conserved.

Histone modifications and VRN1

In winter wheat and barley, initiation of reproductive development is mediated by stable induction of *VRN1* (Diallo, et al., 2012; Oliver, et al., 2009). A previous study indicates that vernalization results in the changes of *VRN1* chromatin state, which includes the increased levels of active marks H3K4me3 and decreased levels of silent marks H3K27me3 (Oliver, et al., 2009). In this study, elevated levels of *AsVRN1* prompted us to examine the transcript levels of H3K4 and H3K27 methyltransferases in TG vs. WT plants. Quantitative RT-PCR analysis shows that H3K27 methyltransferase gene *AsEZ1a* is repressed in transgenic lines, which might release the repression on *AsVRN1* chromatin. Interestingly, levels of two H3K4 methyltransferase genes *AsATX2* and *AsTrx1* are down- and up-regulated in three transgenic lines compared to WT controls. Thus, the hypothesis that *AsVRN1* in TG plants is induced through the elevated active marks of H3K4 methyltransferases cannot be confirmed. It is likely that the chromatin state of other genes is also affected in miR396 TG plants. Therefore, the

validated conclusion cannot be made through analyzing the transcript levels of H3K4 and H3K27 methyltransferase genes. Direct analysis on the relative abundance of H3K4me3 and H3K27me3 would demonstrate the histone modification state of *AsVRN1*. Unfortunately, due to the unavailable genomic information in creeping bentgrass, ChIP-seq analysis for examining the relative abundance of histone modifications is difficult to conduct.

Histone modifications in response to vernalization have been well studied in Arabidopsis. Vernalization leads to the stable repression of *AtFLC* through an increase in silent marks of H3K27me2, H3K27me3, H3K9me2, and H4 arginine 3 dimethylation, and a decrease in active marks of H3K4me2, H3K4me3, and histone acetylation on *FLC* chromatin (Bastow, et al., 2004; Jean Finnegan, et al., 2005; Finnegan and Dennis, 2007; Schmitz, et al., 2008). Among DEGs in TG vs. WT of this study, histone modification-related GO terms were significantly enriched, such as histone acetylation, histone H3K9 methylation regulation of gene expression, and histone arginine methylation. The result suggests that in addition to H3K27 and H3K4 tri-methylation, creeping bentgrass responding to vernalization might recruit other mechanism of histone modification as what has been revealed in Arabidopsis.

Thus far, some evidence has shown that the miR396-GRF module might be involved in histone modifications. A previous study indicates that target GRFs contain a conserved QLQ (glutamine, leucine, and glutamine) motif, which is implicated in chromatin remodeling in the SWI2/SNF2 protein genes of *Saccharomyces cerevisiae* (Treich, et al., 1995). In addition, overexpression of the maize (*Z. mays*) *GRF* gene *ZmGRF10* leads to

the down-regulation of several genes encoding chromatin-modifying proteins (Wu, et al., 2014). A GIF that forms a complex with GRF to regulate downstream transcription activities binds to various chromatin remodeling proteins as well (Vercruyssen, et al., 2014). A recent study indicates that a H3K9 demethylase gene *OsJMJ706* involved in floral organ development is regulated by the *OsmiR396d-OsGRF6*/*OsGRF10* module in rice (Liu, et al., 2014). It is plausible that miR396-GRF module is also involved in vernalization pathway in creeping bentgrass or other cereals. Besides *GRFs*, *SHORT VEGETATIVE PHASE* (*SVP*) has been identified as a target of miR396 in Arabidopsis (Yang, et al., 2015). AtSVP is a MADS-box TF, which forms a complex with AtFLC to repress the expression of *AtFT* and thereby controls flowering time. To control the proper time for floral organ patterning, *AtSVP* also silences the chromatin of a floral organogenesis-related gene *SEPALLATA3* through modulating H3K27me3 (Liu, et al., 2009a). Thus, it is likely that miR396-SVP module is also involved in histone modification in cereals to control flowering time. Validation of SVP as a target of miR396 and investigation of its role in histone modification in creeping bentgrass or other cereals would provide evidence of miR396-mediated flowering time control.

In summary, this work shows that male sterility and plant response to vernalization are dependent on the miR396c-GRF pathway or other miR396c-mediated mechanisms in the perennial monocot species, creeping bentgrass. The male sterility trait may be of use for the genetic hybridization in breeding. Elimination of the vernalization requirement for flowering could provide benefits on expanding area for agriculture practice, avoiding unfavorable environments, and accelerating breeding process.

4.4 Materials and methods

Plant growth

Wild type (WT) and the regenerated transgenic (TG) creeping bentgrass plants were clonally propagated and grown in plastic pots (15 × 10.5 cm, Dillen Products) filled with commercial nutrient-rich soil (3-B Mix, Fafard). The plants were fertilized weekly with 0.2g/L 20:10:20 water-soluble fertilizer (Peat-Lite Special; The Scotts Company) and were maintained in a growth room with SD light regime (14-h of light/ 10-h of dark). Temperatures in the SD growth room were 25°C during the light period and 17°C during the dark period with 350-450 μmol m^{-2} s^{-1} light intensity. The conditions for the LD growth room were the same as the SD growth room, except that the light regime is 16-h of light/8-h of dark. The vernalization treatment was performed in a cold room at 5 °C in an 8-h-ligh/16-h-dark photoperiod. Plants were grown under fluorescent bulbs and the light intensity was 100-150 μmol m^{-2} s^{-1} at plant level. WT and TG plants were propagated at the same time and from the same number of tillers to make sure that they were at the same developmental stage before LD induction and vernalization treatment. We rotated plants every other day to minimize the difference of light intensities on plant growth within each growth room.

Microscopic observations

Spikelets, floralets, anthers, and pollens of WT and TG plants were observed under a stereo microscope (MEIJI EM-5 and MEIJI EMZ-5TR). Photographs were taken as

described in Chapter II. Spikelets and florets of vernalized WT and TG plants at 6th and 8th week after LD induction were detached for observation (Figure 4.2g-j). WT and TG anthers were detached and observed one-day before dehiscence (Figure 4.2k). To compare WT and TG pollen viability (Figure 4.2m, n), pollen was taken out of WT and TG anthers during the highest pollen viability rate of creeping bentgrass (9:00 AM) and stained with 2% (m/v) potassium iodide for microscopic observation (Abràmoff, et al., 2004b; Fei and Nelson, 2003).

Phylogenetic Analysis

The sequence alignments were performed by using the amino acid sequences of the complete the proteins AsVRN1, AsVRN2, and AsVRN3 identified in this study and their orthologs in rice, Arabidopsis, wheat, barley, and Brachypodium based on previous study (Ream, et al., 2012; Higgins, et al., 2010). Phylogenetic trees were generated from the aligned sequences by using the neighbor jointing method in MEGA 6 (Tamura, et al., 2013). The confidence values for the nodes were derived from 1,000 bootstrap replicates. The conserved domains of *VRN1*, *VRN2*, and *VRN3* were aligned by using the BioEdit sequence alignment editor (Hall, 1999).

Plant RNA isolation and expression analysis

Plant sample collection, RNA isolation, semi-quantitative RT-PCR, real-time RT-PCR, stem-loop RT-PCR analyses were as described in Chapter III of this dissertation.

RNA-seq analysis

The cDNA library preparation and Illumina sequencing protocol, differential expression analysis, and GO enrichment analysis were as described previously in Chapter III of this dissertation. GO categories containing less than or equal to five genes were filtered out. The scatterplot of GO enrichment analysis in Figure 4.8 was generated by RVIGO web server (http://revigo.irb.hr/; (Supek, et al., 2011).

4.5 Acknowledgements

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References

Abe M, Kobayashi Y, Yamamoto S, Daimon Y, Yamaguchi A, Ikeda Y, Ichinoki H, Notaguchi M, Goto K, Araki T (2005) FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. Science **309:** 1052-1056

Abràmoff MD, Magalhães PJ, Ram SJ (2004) Image processing with ImageJ. Biophoton Int **11:** 36-42

Amasino R (2010) Seasonal and developmental timing of flowering. Plant J **61:** 1001-1013

Aukerman MJ, Sakai H (2003) Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes. Plant Cell **15:** 2730-2741

Bastow R, Mylne JS, Lister C, Lippman Z, Martienssen RA, Dean C (2004) Vernalization requires epigenetic silencing of FLC by histone methylation. Nature **427:** 164-167

Baucher M, Moussawi J, Vandeputte OM, Monteyne D, Mol A, Pérez-Morga D, El Jaziri M (2013) A role for the miR396/GRF network in specification of organ type during flower development, as supported by ectopic expression of *Populus trichocarpa* miR396c in transgenic tobacco. Plant Biol **15:** 892-898

Behringer C, Schwechheimer C (2015) B-GATA transcription factors–insights into their structure, regulation, and role in plant development. Front Plant Sci **6:**90

Bergonzi S, Albani MC, Ver Loren van Themaat E, Nordstrom KJ, Wang R, Schneeberger K, Moerland PD, Coupland G (2013) Mechanisms of age-dependent response to winter temperature in perennial flowering of *Arabis alpina*. Science **340:** 1094-1097

Chen A, Dubcovsky J (2012) Wheat TILLING mutants show that the vernalization gene VRN1 down-regulates the flowering repressor VRN2 in leaves but is not essential for flowering. PLoS Genet **8**:e1003134

Choi K, Kim J, Hwang HJ, Kim S, Park C, Kim SY, Lee I (2011) The FRIGIDA complex activates transcription of FLC, a strong flowering repressor in Arabidopsis, by recruiting chromatin modification factors. Plant Cell **23:** 289-303

Chu CG, Tan CT, Yu GT, Zhong S, Xu SS, Yan L (2011) A Novel Retrotransposon Inserted in the Dominant Vrn-B1 Allele Confers Spring Growth Habit in Tetraploid Wheat (*Triticum turgidum* L.). G3 (Bethesda) **1:** 637-645

Corley SB, Carpenter R, Copsey L, Coen E (2005) Floral asymmetry involves an interplay between TCP and MYB transcription factors in Antirrhinum. Proc Natl Acad Sci USA **102:** 5068-5073

Danyluk J, Kane NA, Breton G, Limin AE, Fowler DB, Sarhan F (2003) TaVRT-1, a putative transcription factor associated with vegetative to reproductive transition in cereals. Plant Physiol **132:** 1849-1860

Debernardi JM, Mecchia MA, Vercruyssen L, Smaczniak C, Kaufmann K, Inze D, Rodriguez RE, Palatnik JF (2014) Post-transcriptional control of GRF transcription factors by microRNA miR396 and GIF co-activator affects leaf size and longevity. Plant J **79:** 413-426

Debernardi JM, Rodriguez RE, Mecchia MA, Palatnik JF (2012) Functional specialization of the plant miR396 regulatory network through distinct microRNA–target interactions. PLoS Genet **8**:e1002419

Diallo AO, Ali-Benali MA, Badawi M, Houde M, Sarhan F (2012) Expression of vernalization responsive genes in wheat is associated with histone H3 trimethylation. Mol Genet Genomics **287:** 575-590

Distelfeld A, Li C, Dubcovsky J (2009a) Regulation of flowering in temperate cereals. Curr Opin Plant Biol **12:** 178-184

Distelfeld A, Tranquilli G, Li C, Yan L, Dubcovsky J (2009b) Genetic and molecular characterization of the VRN2 loci in tetraploid wheat. Plant Physiol **149:** 245-257

Dubcovsky J, Loukoianov A, Fu D, Valarik M, Sanchez A, Yan L (2006) Effect of photoperiod on the regulation of wheat vernalization genes VRN1 and VRN2. Plant Mol Biol **60:** 469-480

Fei S, Nelson E (2003) Estimation of pollen viability, shedding pattern, and longevity of creeping bentgrass on artificial media. Crop Sci **43:** 2177-2181

Figueroa P, Browse J (2015) Male sterility in Arabidopsis induced by overexpression of a MYC5-SRDX chimeric repressor. Plant J **81:** 849-860

Finnegan EJ, Dennis ES (2007) Vernalization-induced trimethylation of histone H3 lysine 27 at FLC is not maintained in mitotically quiescent cells. Current Biology **17:** 1978-1983

Fu D, Szűcs P, Yan L, Helguera M, Skinner JS, Von Zitzewitz J, Hayes PM, Dubcovsky J (2005) Large deletions within the first intron in VRN-1 are associated with spring growth habit in barley and wheat. Mol Genet Genomics **273:** 54-65

Gonzalez N, De Bodt S, Sulpice R, Jikumaru Y, Chae E, Dhondt S, Van Daele T, De Milde L, Weigel D, Kamiya Y, Stitt M, Beemster GT, Inze D (2010) Increased leaf size: different means to an end. Plant Physiol **153:** 1261-1279

Greenup A, Peacock WJ, Dennis ES, Trevaskis B (2009) The molecular biology of seasonal flowering-responses in Arabidopsis and the cereals. Ann Bot **103:** 1165-1172

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser **41:** 95-98

Heide O (1994) Control of flowering and reproduction in temperate grasses. New Phytol **128:** 347-362

Higgins JA, Bailey PC, Laurie DA (2010) Comparative genomics of flowering time pathways using *Brachypodium distachyon* as a model for the temperate grasses. PLoS One **5:** e10065

Huang F, Xu G, Chi Y, Liu H, Xue Q, Zhao T, Gai J, Yu D (2014) A soybean MADS-box protein modulates floral organ numbers, petal identity and sterility. BMC Plant Biol **14:** 89-2229-14-89

Ito S, Song YH, Josephson-Day AR, Miller RJ, Breton G, Olmstead RG, Imaizumi T (2012) FLOWERING BHLH transcriptional activators control expression of the photoperiodic flowering regulator CONSTANS in Arabidopsis. Proc Natl Acad Sci USA **109:** 3582-3587

Jaudal M, Yeoh CC, Zhang L, Stockum C, Mysore KS, Ratet P, Putterill J (2013) Retroelement insertions at the Medicago FTa1 locus in spring mutants eliminate vernalisation but not long-day requirements for early flowering. Plant J **76:** 580-591

Jean Finnegan E, Kovac KA, Jaligot E, Sheldon CC, James Peacock W, Dennis ES (2005) The downregulation of FLOWERING LOCUS C (FLC) expression in plants with low levels of DNA methylation and by vernalization occurs by distinct mechanisms. Plant J **44:** 420-432

Jones-Rhoades MW, Bartel DP (2004) Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Mol Cell **14:** 787-799

Jung JH, Seo YH, Seo PJ, Reyes JL, Yun J, Chua NH, Park CM (2007) The GIGANTEA-regulated microRNA172 mediates photoperiodic flowering independent of CONSTANS in Arabidopsis. Plant Cell **19:** 2736-2748

Kim JH, Choi D, Kende H (2003) The AtGRF family of putative transcription factors is involved in leaf and cotyledon growth in Arabidopsis. Plant J **36:** 94-104

Kim S, Choi K, Park C, Hwang HJ, Lee I (2006) SUPPRESSOR OF FRIGIDA4, encoding a C2H2-Type zinc finger protein, represses flowering by transcriptional activation of Arabidopsis FLOWERING LOCUS C. Plant Cell **18:** 2985-2998

Kim SY, Michaels SD (2006) SUPPRESSOR OF FRI 4 encodes a nuclear-localized protein that is required for delayed flowering in winter-annual Arabidopsis. Development **133:** 4699-4707

Lee Y, Lee D, Cho L, An G (2014) Rice miR172 induces flowering by suppressing OsIDS1 and SNB, two AP2 genes that negatively regulate expression of Ehd1 and florigens. Rice **7:** 1-13

Li C, Dubcovsky J (2008) Wheat FT protein regulates VRN1 transcription through interactions with FDL2. Plant J **55:** 543-554

Liang G, He H, Li Y, Wang F, Yu D (2014) Molecular mechanism of microRNA396 mediating pistil development in Arabidopsis. Plant Physiol **164:** 249-258

Liu C, Xi W, Shen L, Tan C, Yu H (2009a) Regulation of floral patterning by flowering time genes. Developmental Cell **16:** 711-722

Liu D, Song Y, Chen Z, Yu D (2009b) Ectopic expression of miR396 suppresses GRF target gene expression and alters leaf growth in Arabidopsis. Physiol Plantarum **136:** 223-236

Liu H, Guo S, Xu Y, Li C, Zhang Z, Zhang D, Xu S, Zhang C, Chong K (2014) OsmiR396d-regulated OsGRFs function in floral organogenesis in rice through binding to their targets OsJMJ706 and OsCR4. Plant Physiol **165:** 160-174

Luo Y, Guo Z, Li L (2013) Evolutionary conservation of microRNA regulatory programs in plant flower development. Dev Biol **380:** 133-144

Michaels SD, Amasino RM (1999) FLOWERING LOCUS C encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell **11:** 949-956

Mitsuda N, Seki M, Shinozaki K, Ohme-Takagi M (2005) The NAC transcription factors NST1 and NST2 of Arabidopsis regulate secondary wall thickenings and are required for anther dehiscence. Plant Cell **17:** 2993-3006

Nag A, Jack T (2010) Chapter twelve-sculpting the flower; the role of microRNAs in flower development. Curr Top Dev Biol **91:** 349-378

Ning YQ, Ma ZY, Huang HW, Mo H, Zhao TT, Li L, Cai T, Chen S, Ma L, He XJ (2015) Two novel NAC transcription factors regulate gene expression and flowering time by associating with the histone demethylase JMJ14. Nucleic Acids Res **43:** 1469-1484

Niu L, Lin H, Zhang F, Watira TW, Li G, Tang Y, Wen J, Ratet P, Mysore KS, Tadege M (2015) LOOSE FLOWER, a WUSCHEL-like Homeobox gene, is required for lateral fusion of floral organs in *Medicago truncatula*. Plant J **81:** 480-492

Oliver SN, Finnegan EJ, Dennis ES, Peacock WJ, Trevaskis B (2009) Vernalization-induced flowering in cereals is associated with changes in histone methylation at the VERNALIZATION1 gene. Proc Natl Acad Sci USA **106:** 8386-8391

Omidbakhshfard MA, Proost S, Fujikura U, Mueller-Roeber B (2015) Growth-Regulating Factors (GRFs): A Small Transcription Factor Family with Important Functions in Plant Biology. Molecular plant **8**: 998-1010

Preston JC, Kellogg EA (2008) Discrete developmental roles for temperate cereal grass VERNALIZATION1/FRUITFULL-like genes in flowering competency and the transition to flowering. Plant Physiol **146:** 265-276

Ream TS, Woods DP, Amasino RM (2012) The molecular basis of vernalization in different plant groups. Cold Spring Harb Symp Quant Biol **77:** 105-115

Ream TS, Woods DP, Schwartz CJ, Sanabria CP, Mahoy JA, Walters EM, Kaeppler HF, Amasino RM (2014) Interaction of photoperiod and vernalization determines flowering time of *Brachypodium distachyon.* Plant Physiol **164:** 694-709

Rodriguez RE, Mecchia MA, Debernardi JM, Schommer C, Weigel D, Palatnik JF (2010) Control of cell proliferation in *Arabidopsis thaliana* by microRNA miR396. Development **137:** 103-112

Sasani S, Hemming MN, Oliver SN, Greenup A, Tavakkol-Afshari R, Mahfoozi S, Poustini K, Sharifi HR, Dennis ES, Peacock WJ, Trevaskis B (2009) The influence of vernalization and daylength on expression of flowering-time genes in the shoot apex and leaves of barley (*Hordeum vulgare*). J Exp Bot **60:** 2169-2178

Schmitz RJ, Hong L, Michaels S, Amasino RM (2005) FRIGIDA-ESSENTIAL 1 interacts genetically with FRIGIDA and FRIGIDA-LIKE 1 to promote the winter-annual habit of *Arabidopsis thaliana*. Development **132:** 5471-5478

Schmitz RJ, Sung S, Amasino RM (2008) Histone arginine methylation is required for vernalization-induced epigenetic silencing of FLC in winter-annual *Arabidopsis thaliana*. Proc Natl Acad Sci USA **105:** 411-416

Searle I, He Y, Turck F, Vincent C, Fornara F, Krober S, Amasino RA, Coupland G (2006) The transcription factor FLC confers a flowering response to vernalization by repressing meristem competence and systemic signaling in Arabidopsis. Genes Dev **20:** 898-912

Singh VK, Garg R, Jain M (2013) A global view of transcriptome dynamics during flower development in chickpea by deep sequencing. Plant biotechnology journal **11:** 691-701

Smaczniak C, Immink RG, Angenent GC, Kaufmann K (2012) Developmental and evolutionary diversity of plant MADS-domain factors: insights from recent studies. Development **139:** 3081-3098

Stommel JR, Lightbourn GJ, Winkel BS, Griesbach RJ (2009) Transcription factor families regulate the anthocyanin biosynthetic pathway in *Capsicum annuum*. J Am Soc Hort Sci **134:** 244-251

Sung S, Amasino RM (2004) Vernalization in Arabidopsis thaliana is mediated by the PHD finger protein VIN3. Nature **427:** 159-164

Supek F, Bošnjak M, Škunca N, Šmuc T (2011) REVIGO summarizes and visualizes long lists of gene ontology terms. PloS One **6:** e21800

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol **30:** 2725-2729

Treich I, Cairns BR, de los Santos T, Brewster E, Carlson M (1995) SNF11, a new component of the yeast SNF-SWI complex that interacts with a conserved region of SNF2. Mol Cell Biol **15:** 4240-4248

Trevaskis B, Hemming MN, Peacock WJ, Dennis ES (2006) HvVRN2 responds to daylength, whereas HvVRN1 is regulated by vernalization and developmental status. Plant Physiol **140:** 1397-1405

Turner A, Beales J, Faure S, Dunford RP, Laurie DA (2005) The pseudo-response regulator Ppd-H1 provides adaptation to photoperiod in barley. Science **310:** 1031-1034

Vercruyssen L, Verkest A, Gonzalez N, Heyndrickx KS, Eeckhout D, Han SK, Jegu T, Archacki R, Van Leene J, Andriankaja M, De Bodt S, Abeel T, Coppens F, Dhondt S, De Milde L, Vermeersch M, Maleux K, Gevaert K, Jerzmanowski A, Benhamed M, Wagner D, Vandepoele K, De Jaeger G, Inze D (2014) ANGUSTIFOLIA3 binds to SWI/SNF chromatin remodeling complexes to regulate transcription during Arabidopsis leaf development. Plant Cell **26:** 210-229

Wu G, Park MY, Conway SR, Wang J, Weigel D, Poethig RS (2009) The sequential action of miR156 and miR172 regulates developmental timing in Arabidopsis. Cell **138:** 750-759

Wu L, Zhang D, Xue M, Qian J, He Y, Wang S (2014) Overexpression of the maize GRF10, an endogenous truncated growth-regulating factor protein, leads to reduction in leaf size and plant height. Journal of integrative plant biology **56:** 1053-1063

Wu G, Poethig RS (2006) Temporal regulation of shoot development in *Arabidopsis thaliana* by miR156 and its target SPL3. Development **133:** 3539-3547

Wu L, Liu D, Wu J, Zhang R, Qin Z, Liu D, Li A, Fu D, Zhai W, Mao L (2013) Regulation of FLOWERING LOCUS T by a microRNA in *Brachypodium distachyon*. Plant Cell **25:** 4363-4377

Yan L, Fu D, Li C, Blechl A, Tranquilli G, Bonafede M, Sanchez A, Valarik M, Yasuda S, Dubcovsky J (2006) The wheat and barley vernalization gene VRN3 is an orthologue of FT. Proc Natl Acad Sci USA **103:** 19581-19586

Yan L, Loukoianov A, Blechl A, Tranquilli G, Ramakrishna W, SanMiguel P, Bennetzen JL, Echenique V, Dubcovsky J (2004) The wheat VRN2 gene is a flowering repressor down-regulated by vernalization. Science **303:** 1640-1644

Yan L, Loukoianov A, Tranquilli G, Helguera M, Fahima T, Dubcovsky J (2003) Positional cloning of the wheat vernalization gene VRN1. Proc Natl Acad Sci U S A **100:** 6263-6268

Yang F, Liang G, Liu D, Yu D (2009) Arabidopsis miR396 mediates the development of leaves and flowers in transgenic tobacco. Journal of Plant Biology **52:** 475-481

Yang C, Xu Z, Song J, Conner K, Vizcay Barrena G, Wilson ZA (2007) Arabidopsis MYB26/MALE STERILE35 regulates secondary thickening in the endothecium and is essential for anther dehiscence. Plant Cell **19:** 534-548

Yang CY, Huang YH, Lin CP, Lin YY, Hsu HC, Wang CN, Liu LY, Shen BN, Lin SS (2015) MicroRNA396-Targeted SHORT VEGETATIVE PHASE Is Required to Repress Flowering and Is Related to the Development of Abnormal Flower Symptoms by the Phyllody Symptoms1 Effector. Plant Physiol **168:** 1702-1716

Yuan S, Li Z, Li D, Yuan N, Hu Q, Luo H (2015) Constitutive Expression of Rice MicroRNA528 Alters Plant Development and Enhances Tolerance to Salinity Stress and Nitrogen Starvation in Creeping Bentgrass. Plant Physiol **169:** 576-593

Zhang J, Wang Y, Wu S, Yang J, Liu H, Zhou Y (2012) A single nucleotide polymorphism at the Vrn-D1 promoter region in common wheat is associated with vernalization response. Theor Appl Genet **125:** 1697-1704

Zhao X, Harashima H, Dissmeyer N, Pusch S, Weimer AK, Bramsiepe J, Bouyer D, Rademacher S, Nowack MK, Novak B (2012) A general G1/S-phase cell-cycle control module in the flowering plant *Arabidopsis thaliana*. PLoS Genet **8:** e1002847

Zhou CM, Zhang TQ, Wang X, Yu S, Lian H, Tang H, Feng ZY, Zozomova-Lihova J, Wang JW (2013) Molecular basis of age-dependent vernalization in *Cardamine flexuosa*. Science **340:** 1097-1100

CHAPTER V - CONCLUSION AND FUTURE DIRECTIONS

Plant leaf morphology, tillering, floral organ development, flowering time control, and abiotic stress tolerance are important agronomic traits for genetic modification to improve crop production. I have investigated the feasibility of manipulating miRNAs for trait modifications in a perennial monocot species, creeping bentgrass.

MiR528 is a conserved monocot-specific small RNA that is potentially involved in multiple environmental stresses. I generated transgenic creeping bentgrass overexpressing *Osa-miR528* and compared plant development and abiotic stress tolerance between WT and TG plants. We are the first to demonstrate that miR528 regulates shoot architecture. Transgenics display the desirable turf trait of the dwarf and dense phenotype due to increased tiller number, reduced internodes and tiller length. We are also the first to experimentally validate that miR528 enhances high salinity and N deficiency tolerance. During high salinity exposure, TG plants show improved K^+ retention, up-regulated gene expression of the K^+ transporter *AsHAK5*, and enhanced ROS scavenging associated with increased CAT activity but decreased AAO activity. The improved N deficiency tolerance results from enhanced ROS scavenging and NUE associated with up-regulated gene expression and increased enzyme activity of nitrite reductase. In addition, two putative miR528 targets, *AsCBP1* and *AsAAO* have been identified in creeping bentgrass. To investigate if the targets directly contribute to the enhanced stress tolerance, I analyzed their expression profiles and found that both targets are induced under salt and N deficiency conditions in WT leaves. Further molecular analysis shows

that miR528 has cross talk with other abiotic stress-related small RNAs and transcription factors in perennial grasses.

Our results demonstrate the importance of miR528 in plant development and its key role as a multistress integrator regulating plant responses to high salinity and N starvation, pointing to the potential of manipulating miR528 in improving plant abiotic stress resistance. Thus far, the molecular mechanism of miR528-mediated plant development remains unclear. Identifying more targets of miR528 will gain insight into the central role of miR528 in the complex regulatory network. Additionally, the role of miR528-AAO and miR528-CBP1 pathways have not been characterized individually. To avoid pleiotropic phenotypes and to better manipulate miR528-target module for plant improvement, it is critical to functionally characterize the targets of miR528.

Another conserved small RNA miR396 is also largely involved in plant development and abiotic stress response. Thus far, miR396-mediated plant development and abiotic stress response have not been studied in perennial grasses. In this study, I generated transgenic creeping bentgrass overexpressing a rice miRNA, *Osa-miR396c*. During vegetative growth, transgenics display altered development, including less shoot and root biomass, shorter internodes, smaller leaf size, fewer leaf veins and epidermis cells than those of WT controls. To analyze what causes the altered vegetative growth, we identified four miR396 putative targets, *AsGRF3-6*, whose homologues are critical in controlling cell proliferation. These four targets show repressed expression in TG plants, contributing to the altered development during vegetative growth. In addition, RNA-seq

analysis indicates that DEGs involved in cell division are significantly enriched and down-regulated, which also leads to altered plant development. During high salinity exposure, TG plants show enhanced salt tolerance associated with less accumulation of Na^+ in leaves than in that of the WT controls, which is possibly attributed to a Na^+ exclusion mechanism. Further analysis of the expression levels of the Na^+/H^+ antiporter *AsSOS1* confirmed our hypothesis. To detect if miR396 targets are directly involved in salt stress response, we analyze their expression patterns. Our data show that *AsGRF3-6* genes are all induced under salt stress treatment, suggesting that miR396 might mediate plant salt stress tolerance through regulating its targets. In addition, GO enrichment analysis demonstrates that transcription factors, protein kinases, and antioxidant enzymes are significantly enriched, which leads to salt stress tolerance and salt stress-induced oxidative stress tolerance.

Our data establishes a molecular pathway to connect the upstream regulatory and downstream functional stress-responsive elements, and gains insight into a miR396-mediated regulatory network in plant abiotic stress response. Identification and characterization of more GRFs and grass-specific targets of miR396 and downstream targets of GRFs will provide a better understanding of the role of specific targets and the regulatory network.

In addition to plant development during vegetative growth and abiotic stress response, study on plant reproductive growth indicates that miR396 is also involved in floral organ development and flowering time control. We are the first to explore that miR396 controls

floral organ development and male viability in perennial grasses. TG plants display male sterility, which includes the defects in filament elongation, anther dehiscence, and pollen viability. Repressed expression of *AsGRF3-6* in TG plants contributes to the altered floral organ development. Genome-wide analysis shows that GO terms of 'floral organ formation', 'anther dehiscence', 'starch metabolic process' etc. are significantly enriched, which also leads to changes in floral organs. Interestingly, we found that miR396 TG plants flower without a vernalization requirement. This is the first report about the involvement of miR396 in flowering time control or vernalization pathway. We cloned the key genes, *AsVRN1-3* in the vernalization pathway and analyzed their expression patterns under SD-LD and SD-cold-LD conditions. The differential expression levels of *AsVRN1* under SD conditions prompt us to hypothesize that miR396 indirectly activates *AsVRN1*, and thus leading to the elimination of the vernalization requirement. Since histone modifications activate *VRN1* in wheat and barley, we analyzed the expression levels of methyltransferase genes in WT and TG plants. We found that genes encoding methyltransferases show different expression patterns in WT and TG plants. In addition, histone modification-related GO terms are significantly enriched in DEGs of TG vs. WT plants.

To our knowledge, this is the first report demonstrating the involvement of miR396 in flowering time control. To validate if the activation of *AsVRN1* under SD conditions in miR396 TG plants results from the altered histone modification state, direct analysis on the relative abundance of histone silent and active marks is required. Currently, due to the unavailable genomic information of creeping bentgrass, the measurement of the relative

abundance of histone modifications is difficult to conduct. The next step would be to examine the impact of miR396 in controlling plant response to vernalization in other crop species with available genomic information, such as wheat or barley. This would provide information to better understand miR396-mediated flowering time control.

Collectively, my research reveals that various agronomic traits are impacted by small RNAs in perennial grasses. I also examined and validated the feasibility of manipulating miR528 and miR396 to genetically engineer plant for improved plant production.

APPENDIX A- SUPPLEMENTARY FIGURES

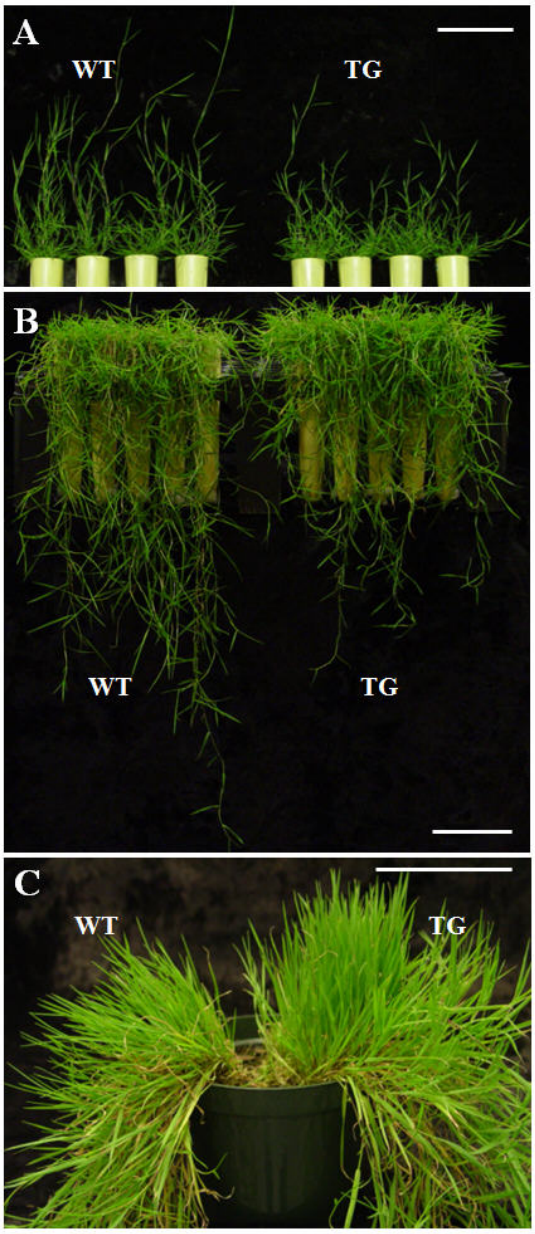


Figure A-1 Development of WT and TG plants. A, Five-week-old WT and TG plants initiated from a single tiller. B, Ten-week-old WT and TG plants initiated from a single tiller. C, Non-elongated tillers of two-month-old WT and TG plants initiated from the same amount of tillers were grown in the same 6-inch pot. Scale bar = 10 cm.

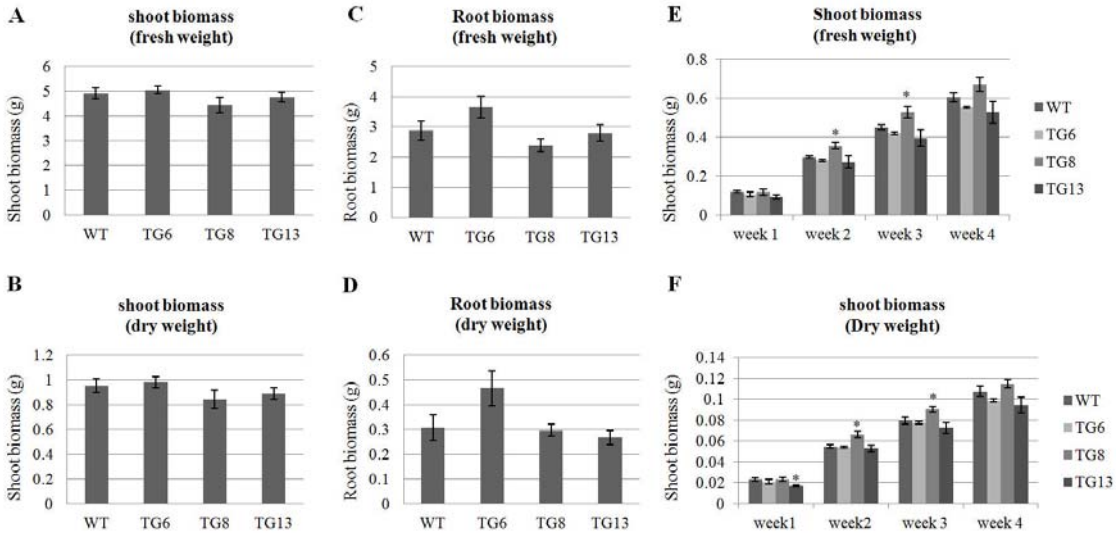


Figure A-2 Biomass measurement in WT and TG plants. A, Shoot fresh weight and B, shoot dry weight of WT and TG plants 10 weeks after initiation from a single tiller (n=4). C, Root fresh weight and D, root dry weight of WT and TG plants 10 weeks after initiation from a single tiller (n=4). E, Fully developed WT and TG plants grown in small cone-tainers were mowed weekly to the same height. Clippings fresh weight and F, dry weight in WT and TG plants were measured every week (n=4). Data are presented as means, and error bars represent ±SE. Asterisk (*) indicates a significant difference of shoot or root biomass between WT and each transgenic line at $P<0.05$ by Student's *t-test*.

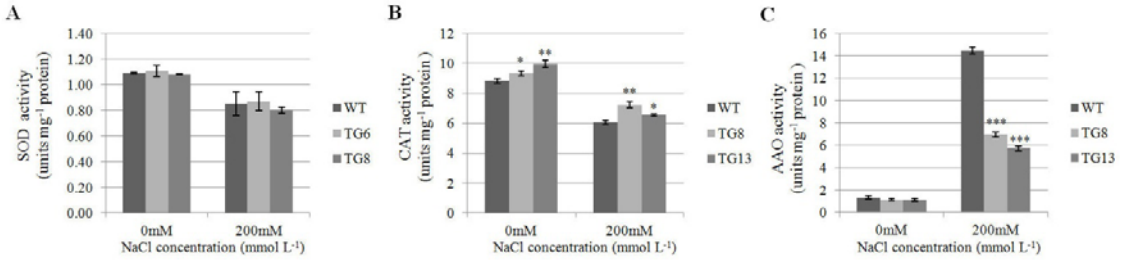


Figure A-3 Antioxidant enzyme activity assay under normal and 200 mM NaCl conditions in WT and TG plants. A, SOD, B, CAT, and C, AAO activity assays of WT and TG leaves before and after 200 mM NaCl treatment. Data are presented as means (n=4), and error bars represent ±SE. Asterisks (*, ** or ***) indicate significant differences of enzyme activity between WT and each transgenic line at $P<0.05$, 0.01 or 0.001 by Student's *t-test*.

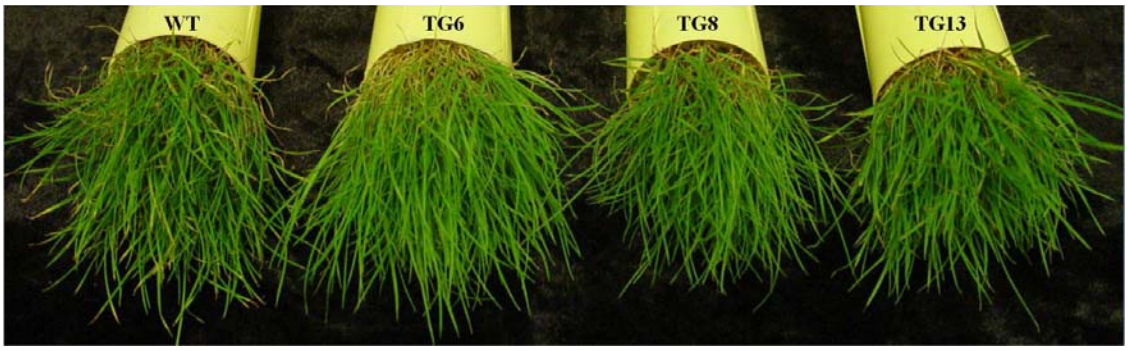


Figure A-4 WT plants exhibit wilting leaf tips compared to three transgenic lines.

Figure A-5 Biomass accumulation of WT and *Osa-miR528*TG plants in response to different concentrations of N solutions.

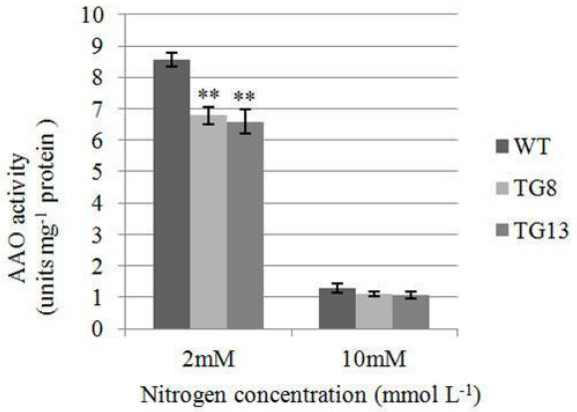
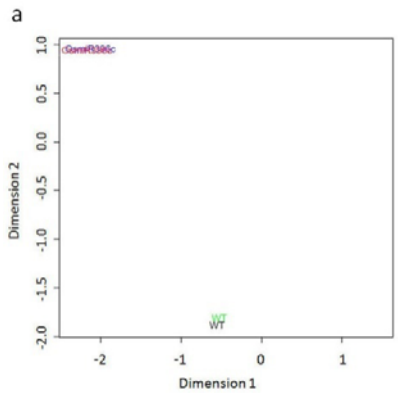
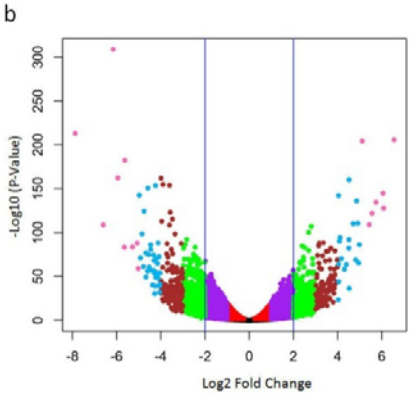


Figure A-6 AAO activity measurement under normal and N deficiency conditions. Data are presented as means (n=3), and error bars represent ±SE. Asterisks (**) indicate significant differences of AAO activity between WT and each transgenic line at $P<0.01$ by Student's *t-test*.





c

Log2 Fold Change	Gene number
logFC > 1	3475
logFC > 2	584
logFC > 3	118
logFC > 4	27
logFC > 5	7
logFC < -1	4767
logFC < -2	1027
logFC < -3	193
logFC < -4	44
logFC < -5	9

Figure A-7 Differential expression of genes in TG vs. WT plants. (a) MDS plot of WT and *Osa-miR396c* TG RNA-seq samples with two biological replicates. (b) Volcano plot shows $\log_2$ FC of TG vs. WT data sets at LD non-stressed conditions. (c) A table shows $\log_2$ FC values and corresponding number of genes.

Figure A-8 Vernalized (+V) and non-vernalized (-V) transgenic *C. flexuosa* overexpressing miR396 under 2-week LD induction. Non-vernalized TG *C. flexuosa* plants were not flowering after LD induction for 15 weeks, while 5-week-old TG plants were flowering after 8-week cold treatment followed by 2-week LD induction.